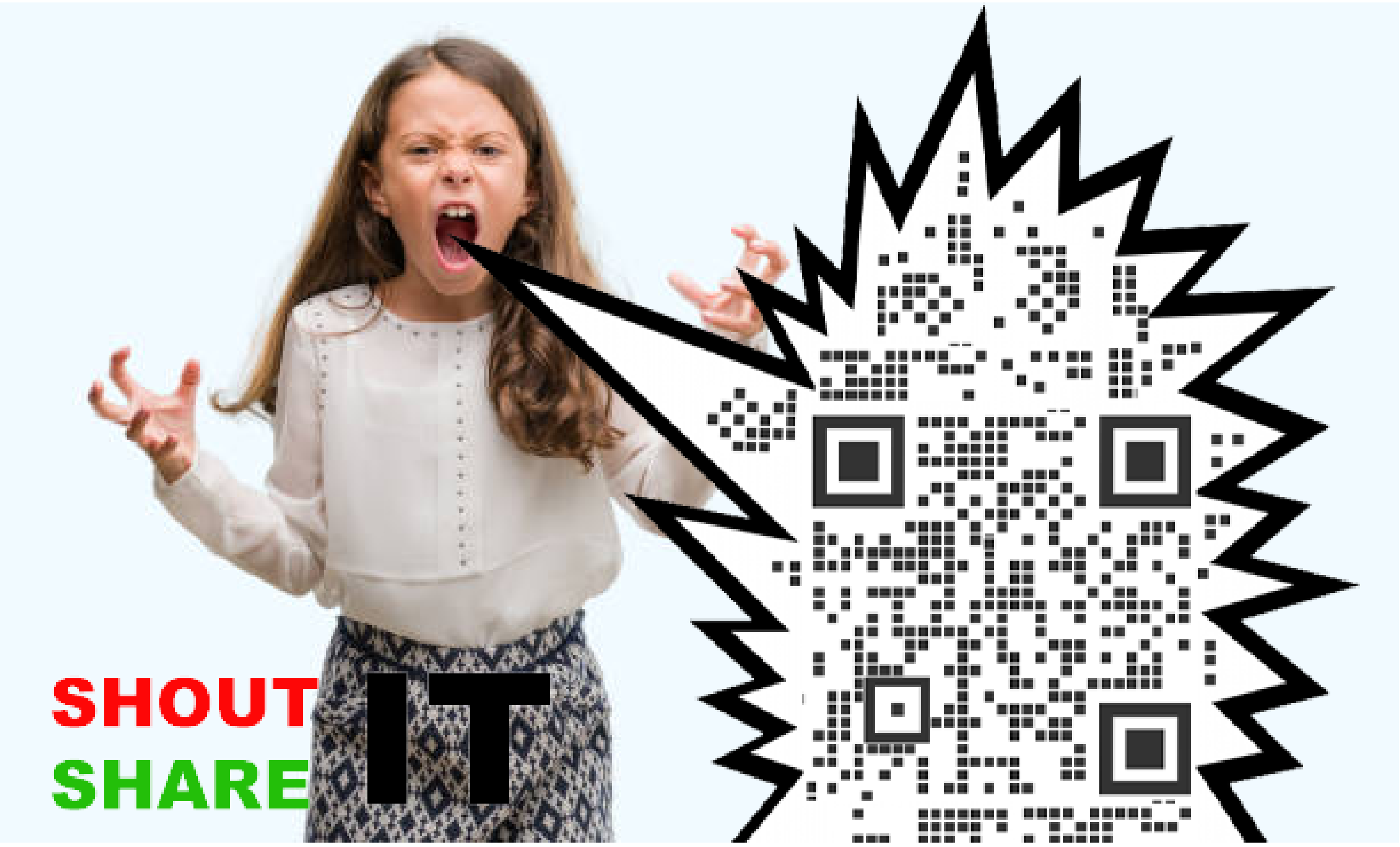
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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

VOL. XLIV JANUARY, 1923 No. 1

CONTENTS

PAGE

JUST, E. E. *The Fertilization-Reaction in Echinarachnius parma, VI. The Necessity of the Egg Cortex for Fertilization* 1

JUST, E. E. *The Fertilization-Reaction in Echinarachnius parma, VII. The Inhibitory Action of Blood* 10

JUST, E. E. *The Fertilization-Reaction in Echinarachnius parma, VIII. Fertilization in Dilute Sea-Water* 17

KEPNER, WM. A., AND REYNOLDS, B. D. *Reactions of Cell-Bodies and Pseudopodial of Fragments of Difflugia* 22

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George T. Moore—*The Missouri Botanic Garden.*
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Contents of Volume XLIV

No. 1. JANUARY, 1923

JUST, E. E. *The Fertilization-Reaction in Echinarachnius parma, VI. The Necessity of the Egg Cortex for Fertilization* 1
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JUST, E. E. *The Fertilization-Reaction in Echinarachnius parma, VIII. Fertilization in Dilute Sea-Water* 17
KEPNER, WM. A. AND REYNOLDS, B. D. *Reactions of Cell-Bodies and Pseudopodial of Fragments of Difflugia* 22

No. 2. FEBRUARY, 1923

LILLIE, FRANK R. *Supplementary Notes on Twins in Cattle*. 47
GLASER, OTTO. *Copper, Enzymes, and Fertilization* 79

No. 3. MARCH, 1923

ZELENY, CHARLES. *The Temperature Coefficient of a Heterozygote with an Expression for the Value of a Germinal Difference in Terms of an Environmental One* 105
SIVICKIS, P. B. *Studies on the Physiology of Reconstitution in Planaria lata, with a Description of the Species* 113

No. 4. APRIL, 1923

MCNAIR, GEORGE T. *Motor Reactions of the Fresh-Water Sponge, Ephydatia fluviatilis* 153
ALLEE, W. C. *Studies in Marine Ecology. I, The Distribution of Common Littoral Invertebrates of the Woods Hole Region* 167
LAMBERT, W. V., RICE, W. S., AND WALKER, H. C. A. *Food and Parthenogenetic Reproduction as Related to the Constitutional Vigor of Hydatina senta* 192

No. 5. May, 1923

Allee, W. C. *Studies in Marine Ecology. III, Some Physical Factors Related to the Distribution of Littoral Invertebrates* 205

No. 6. June, 1923

Hoadley, Leigh. *Certain Effects of the Salts of the Heavy Metals on the Fertilization Reaction in Arbacia punctulata* 255
Watt, James C. *The Behavior of Calcium Phosphate and Calcium Carbonate (Bone Salts) Precipitated in Various Media, with Applications to Bone Formation* 280

Vol. XLIV. January, 1923. No. 1.

BIOLOGICAL BULLETIN

THE FERTILIZATION-REACTION IN *ECHINARACHNIUS PARMA*. VI.

THE NECESSITY OF THE EGG CORTEX FOR FERTILIZATION.

E. E. JUST,[1]

ROSENWALD FELLOW IN BIOLOGY, NATIONAL RESEARCH COUNCIL.

If we define fertilization as an instantaneous irreversible reaction at or in the cortex of the egg between an ovogenous substance, fertilizin, and the spermatozoön, it must follow (1) that an egg, once fertilized, is incapable of response to additional insemination, and (2) that fragments of fertilized eggs are likewise incapable of fertilization. If, moreover, the fertilization-reaction be limited to the cortex, then it must likewise be shown (3) that uninseminated eggs, or fragments thereof, devoid of cortex are not fertilizable. The present paper aims to set forth certain observations made at the Marine Biological Laboratory, Woods Hole, Mass., on the egg of *Echinarachnius parma* which indicate that fertilized eggs, or fragments thereof, are unfertilizable; and that uninseminated eggs, or fragments thereof, devoid of cortex are likewise unfertilizable. It is therefore concluded that the cortex of the uninseminated egg is necessary for fertilization.

II.

The fertilized egg does not react to additional insemination; sperm do not enter fertilized eggs. In order to test the validity of this generally accepted statement, fertilized eggs after removal of their membranes have been inseminated two, three, and four minutes after insemination and at later stages during development to gastrulation. Such eggs have been sectioned.

In no case have sperm been found in the egg or blastomeres after even the heaviest insemination. Rupture of the blastomeres with outflow of cytoplasm does not facilitate sperm entry. To offset the possibility that fixation might be a source of error, most diverse fluids were used. In the living egg, in addition, it was easy to see that spermatozoa do not react with fertilized eggs. There is here, certainly, no evidence in support of Kohlbrugge's results.

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[1] Zoölogical Laboratory, Howard University.

Many experiments have likewise been made thus: Eggs are lightly inseminated and at five-second intervals up to the time of membrane separation are given an additional heavy insemination. Such eggs fail to reveal polyspermy in higher per cent. than eggs that have but one insemination.

Thus, June 28, 1918, eggs of *Echinarachnius* were inseminated and at five-second intervals up to time of membrane separation were reinseminated. After membrane separation, the eggs were gently shaken to remove the membranes; samples of these were reinseminated at six, thirteen, sixteen, and twenty minutes after the original insemination. Samples of these eggs were fixed in corrosive sublimate-acetic two minutes after each insemination. No evidence of a reaction was found in any of the sectioned material.

In addition, the bulk of the evidence (Lillie, '19) shows that artificially activated eggs fail to react with sperm.

Wilson found that fragments of fertilized eggs of *Cerebratulus* are incapable of fertilization. Conklin has shown that the unusually large polar bodies produced by the egg of *Crepidula* through centrifugal force do not fertilize. Since in this egg polar-body formation follows insemination, Conklin's results are capable of the interpretation that here, too, parts of fertilized eggs do not refertilize.

In *Echinarachnius* the situation is the same. If inseminated eggs of *Echinarachnius* be gently shaken in a vial with bits of broken coverslips and the fragments thus obtained be divided into two lots, one of which is inseminated, the per cent. of development in the two lots is the same. The insemination of these fragments, even if made *twenty seconds* after the insemination of the intact eggs, does not increase the per cent. of development.

These observations indicate that fertilization is irreversible, eggs completely activated can not respond to additional insemination; and fragments of inseminated eggs behave similarly.

Through the well-known experiments of the Hertwigs, Boveri, Morgan, and others it has been shown that enucleated fragments from uninseminated eggs of sea-urchins are fertilizable as are nucleated fragments. Wilson has shown that the enucleated fragments taken from the egg of *Cerebratulus* after dissolution of the germinal vesicle are fertilizable. In a very cautiously worded paper appearing posthumously Boveri maintained his original position as to the fertilizability of enucleated fragments of uninseminated eggs.

I find that fragments from uninseminated eggs of *Echinarachnius* obtained by gently shaking the eggs in a vial with bits of a broken coverslip are capable of fertilization and development. The development of these fragments does not depend upon the presence of the egg nucleus. Some fragments without egg nuclei fail to respond to insemination. A fragment of large size and with a nucleus may not fertilize. Very small fragments with no egg nucleus develop. *I believe that the failure of fragments to fertilize is due to the absence of cortical material.* This belief is based on results which may now be considered.

III.

Toward the end of the season of 1917 I frequently found that fertilized eggs of the *Echinarachnius* gave rise to abnormal gastrulæ which I took to be ordinary exogastrulæ. They prove to be gastrulæ with masses of undifferentiated protoplasm attached.[1] The breaking up of these masses simulates cleavage. A careful study of these eggs was made and the history of this condition revealed.

I found that among various lots of eggs kept for some time in shallow dishes with little sea-water were some eggs which on return to a larger quantity of normal sea-water underwent a fragmentation. Under the microscope this process is easily followed. The eggs give off a bud or form an exovate that slowly increases in size and drops off. Thus in a given lot of eggs there are innumerable cases each with a bud of varying size still attached to or just detached from the egg. If insemination takes place before the bud drops off, a membrane separates from the "egg" and never from the bud. Repeated observation puts this statement beyond doubt. I have never seen two membranes on such eggs, nor a single membrane enclosing both egg and bud. The portion within the membrane alone cleaves and develops. The portion outside the membrane never develops; it remains attached to the gastrula until completely disintegrated. In some cases the bud is so much larger than the "egg" that membrane separation takes place from a relatively small disk; the cleavage of such eggs is discoidal; such eggs never give rise to swimmers.

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[1] These masses are always located at the vegetative pole. This may be significant for the problem of polarity.

If the observer inseminate eggs after the buds drop off, only one member of a pair separates a membrane, cleaves, and gastrulates, though it may be the smaller. The presence or absence of the egg nucleus is of no consequence for the development of these fragments.

Any one of three possibilities was thought of as responsible for this phenomenon of bud formation in the egg of *Echinarachnius:* (1) staleness of the eggs, (2) the presence of blood, (3) the general deterioration of the sexual products toward the end of the season. Accordingly, in 1918 attempts were made to ascertain which of these factors is responsible for this bud formation. And we may state at the outset that though each factor may contribute to the production of buds, *the essential factor is the hypertonicity of the medium.*

If eggs of *Echinarachnius* are allowed to stand in sea-water for several hours, they slowly undergo changes that eventually lead to their complete disintegration. A portion of these eggs upon insemination separate membranes many of which are stuck to the swollen cortex. If previous to insemination these eggs be gently shaken, buds are formed from those with swollen cortex. Such inseminated eggs with buds separate membranes only from the "eggs" and never from the buds. These eggs cleave and gastrulate, but the per cent. is always low. Late in the season buds are more easily produced. And throughout the season the presence of blood increases the number of buds formed.

By far the easiest method for the production of a high per cent.

of buds at any time during the season is to allow the uninseminated eggs to stand in a small quantity of sea-water in a shallow dish, thus permitting evaporation; or, better, to place uninseminated eggs in hypertonic sea-water (6 parts of 2½ *M* NaCl plus 50 parts sea-water). On transfer of the eggs to normal sea-water they are gently shaken or squirted through a pipette. Large numbers of such eggs produce buds.

On insemination membranes separate from but one component of these budded eggs. Only that portion of the egg within the membrane divides and gastrulates. The gastrulæ swim attached to the undifferentiated mass of budded cytoplasm which eventually disintegrates. The process of bud formation is easily followed under the microscope and insemination made at any stage. Insemination made after complete separation of the bud gives the same result: in any two given masses of egg material separated by constriction of a bud one only develops, regardless of size or the presence of the egg nucleus.

The explanation of these results on budded eggs of *Echinarachnius* is as follows: The cortex of the eggs changes under various forms of treatment. As the uninseminated egg of *Echinarachnius* lies in sea-water it slowly deteriorates. A distinguishing mark of this deterioration is the physical change in the cortex: the cortex is thick and practically transparent. Late in the season also many eggs are found with thick cortices. Blood, too, will frequently hasten this change in the cortex. Now, hypertonic sea-water very clearly brings about a physical change in the cortex. After exposure to hypertonic sea-water the cortex may be readily seen as a thick jelly-like hull enclosing the egg. It is from this jellied cortex that the membrane separates on insemination.

If an egg with a thick cortex be gently shaken on transferal to normal sea-water, the cortex breaks and the contents of the egg flows out. Indeed, merely the transfer from hypertonic sea-water to normal sea-water will tend to produce this outflow in some eggs, as they rapidly take up water. The bud is thus made up of endoplasm and is without cortical material. In favorable cases this is readily determined.

And only that component of the budded egg which has the clear rim of cortex is fertilized on insemination as revealed by the presence of the membrane, cleavage, and gastrulation. The naked mass of endoplasm rounds up still attached to the developing egg. It never reacts with sperm whether inseminated while attached to the egg or after separated from the egg. Thus the presence of the egg cortex is necessary for fertilization. Many observations make this interpretation certain.

In the egg of *Arbacia* the results are the same; indeed, if anything, they are more clear-cut.

Hypertonic sea-water is not the only agent that will bring about this outflow of endoplasm. Frequently shaking will bring it about in a few eggs of a given lot. Hypertonic sea-water is best, however. first because it produces a high per cent. of budded eggs, and second because it makes very clear that the cortex is on the egg and not on the endoplasmic mass.

One additional method may be mentioned now because its use has in turn led to some interesting experiments along another line. This method involves the use of bolting silk, soft filter paper, and lens paper. We may briefly consider this method.

Uninseminated eggs of *Echinarachnius* are dropped on bolting silk (in focus under low power of the microscope), the mesh of which has a diameter less than that of the egg, stretched above the surface of sea-water in a stender dish. If the concentration of eggs in the drop of sea-water is just right, some eggs rupture as they flow through the meshes of the silk. If the observer work rapidly, he can after trial inseminate these eggs just as they burst. The silk is then quickly thrust into the dish of sea-water. Some of the eggs form membranes with naked buds attached.

With filter paper the method is much the same. Soft moist filter paper on which is placed a drop of eggs is mounted under the microscope above sea-water in a low stender dish. The eggs flow beneath the fibers of the filter paper and thus burst because of pressure and slight drying. As they burst they are inseminated and the paper plunged into sea-water. Some of these eggs later show buds without membranes attached to cleaving eggs within membranes. Intact eggs inseminated among the fibers of filter paper in sea-water on insemination will develop normally. I have kept such eggs through to the pluteus stage. With lens paper one may obtain much the same results; the lens paper, in addition, is much easier to handle: the endoplasmic outflow is more readily followed.

With a little care one may induce flow of endoplasm through the cortex. The naked endoplasm rounds up and in appearance is like the remaining part of the egg. But the endoplasm does not fertilize; it fails to react with sperm.

Here, again, eggs of *Arbacia* give comparable results.

While my observations were under way in 1918, Dr. Robert Chambers informed me that by the method of microdissection he was able to remove the cortex from the egg of the starfish (Lillie, '19). Such eggs are incapable of fertilization. Portions of the egg with cortical material, on the other hand, readily fertilize.

We may conclude from these observations that certain forms of treatment so alter the cortex as to facilitate endoplasmic outflow. By such treatment the fertilization capacity of the egg is not lost; it is, however, localized in only that part of the egg enclosed by cortical material. It thus follows that the inner substance of the egg is non-fertilizable in fertilized eggs not because of progressive centripetal changes set up at the cortex on insemination, but because the endoplasm is inherently non-fertilizable. Again, it is not necessary to postulate that the development of fragments from uninseminated eggs following fertilization may be due to the presence of some nuclear material of the egg (cf. Boveri). If the interpretation of the observations here reported be correct, fragments of uninseminated eggs, whether nucleated or not, are fertilizable if they possess cortical material. The egg cortex is thus necessary for the fertilization-reaction.

IV.

In any attempt at defining fertilization we must consider several facts.

In the first place, animal ova vary with respect to the stage in their development in which they are fertilized. Thus some reach the fertilizable condition before the germinal vesicle breaks down, others in the mesophase of the first maturation, still others during the second maturation, and many after maturation is complete. Starfish eggs may be fertilized at any time from the dissolution of the germinal vesicle to a short time after complete maturation.

Nor, again, is mere sperm penetration fertilization, since sperm normally penetrate ova (*Dinophilus, Saccocirrus,* etc.) some time before fertilization ensues. There are thus all possible types of fertilization with respect to the maturation stage of the egg when normally inseminated. No definition of fertilization is worth while if based on one type of egg alone.

In the second place, though the end result of fertilization is cleavage, there are here, too, many differences among animal ova. Thus the zygote nucleus may at first divide without cytoplasmic division (*Renilla*); the germ nuclei may fuse or appose merely; the cleavage spindle may be homodynamic, or heterodynamic; the sperm amphiaster may be homo- or heterodynamic, its second aster arising before or after union with the egg nucleus; and the cleavage centers may arise about the sperm nucleus or the egg nucleus or in part about each. A definition of fertilization in terms of the behavior of the germ nuclei or of the origin of the cleavage centers is manifestly inadequate.

If, for example, we consider the classic theory of Boveri that fertilization is due to the introduction of centrosomes by the spermatozoön, we realize its inadequacy at once, since it demands that the middle-piece enter the egg. It is true that the whole spermatozoön enters certain eggs whose maturation spindles are without centrosomes or asters, thus apparently supplying a deficiency. But in many other cases the middle-piece does not enter the egg, and where it does as in echinid ova the identity of its so-called centrosomes is wholly mistaken. To support the Boveri hypothesis we must shift the position of the potent centrosomes to fit those cases where the middle-piece does not enter the egg, or on entering takes no part in aster formation.

Because of failure to recall these simple facts purely morphological theories of fertilization fail. Indeed, many studies on fertilization are but studies of cell division; they deal with structures and phenomena in cell division in no wise restricted to egg cells. Nor yet have many physical or chemical theories been more fortunate. These theories are based on the study of physiological changes incident to cell division. But cell division is not fertilization.

An approach to the fertilization problem can be made only through study of fertilization in the most diverse types of ova and by rejection of the incidental phenomena for the basic and common. What is the common factor in fertilization? So far as we know, it is some type of cortical change. But by cortical change we do not mean that the sign of the thing is the thing itself. Thus membrane separation in the sea-urchin egg is an easily visible sign of cortical change. Membrane separation, however, is not fertilization. It is here that an error lies in much of the work on experimental parthenogenesis.

Though we may doubtless gain knowledge of the nature of the cortical changes following insemination through study of these changes experimentally induced, we can not rely wholly on such work to explain fertilization. A far more simple mode of attack is to study fertilization itself. And if, in addition, the theory of the action of the agent in experimental parthenogenesis is erroneous, such a theory for fertilization can but hinder the solution of our problem. If it be true that cell division is not fertilization, it is equally true that experimental parthenogenesis is not fertilization. We must, therefore, study the fertilization process itself, the common factor of which is some kind of cortical change.

The evidence at hand indicates that the cortical changes in fertilization are due to an instantaneous, irreversible reaction between an ovogenous substance, fertilizin, and the spermatozoön. Stated in these terms the theory almost demands that the cortex is necessary for fertilization. The evidence herewith submitted points to this conclusion. The primary, if not, indeed, the whole event in fertilization, is the cortical reaction. The succeeding events with concomitant physical and chemical changes leading to cell division and development are the consequence of a complete cortical reaction between fertilizin and spermatozoön.

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THE FERTILIZATION–REACTION IN *ECHINARACHNIUS PARMA.* VII.

The Inhibitory Action of Blood.

E. E. JUST,[1]

Rosenwald Fellow in Biology, National Research Council.

The present communication aims to set forth results of experiments made during two seasons (1919 and 1920) at the Marine Biological Laboratory, Woods Hole, Mass., to test that part of Lillie's fertilizin theory which postulates that blood (in *Arbacia*) inhibits fertilization through intervention of the fertilizin and the egg (Lillie, '14). The present writer was firmly of the opinion that this postulated action might be merely a surface effect: that despite the agglutination of *Arbacia* sperm, by *Arbacia* egg-water in the presence of specific blood, the main action of the blood is on the surface of the egg so that sperm can not enter. The results of the experiments here reported, however, show that, in the egg of *Echinarachnius parma* at least, this is not the case: blood blocks fertilization in this egg by interfering with the reaction of fertilizin and egg and not with the sperm and fertilizin at the surface of the egg. For repeated observations reveal that both in straight and cross fertilization, with *Arbacia* sperm, eggs of *Echinarachnius* inseminated in blood, though they fail to develop, nevertheless take in sperm. We may divide the experiments into two groups: those that deal with straight fertilization and those that deal with cross fertilization with *Arbacia* sperm.

I.

Eggs of *Echinarachnius* obtained by cutting up ovaries in sea-water invariably give low fertilization percentages. Thus the early observations—1910, 1914, 1915—made on such eggs gave the impression that this is a poor egg for the study of fertilization. An egg suspension strained from ovaries cut up in sea-water shows a slight turbidity or greater depth in color depending upon the amount of blood and detritus present. My notes indicate that fertilizing power falls off with increasing depth of color. With shed eggs, on the other hand, the case is quite different: they invariably yield 100 per cent. fertilization. If, however, shed eggs of maximum fertilization capacity be inseminated in cœlomic fluid, the per cent. of cleavage is decreased. Thus equal parts of cœlomic fluid and sea-water may cut down the per cent. of cleavage to zero; higher proportions of cœlomic fluid, 75 to 100 per cent., invariably permit no fertilization.

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[1] Zoölogical Laboratory, Howard University.

In practice it was found extremely difficult to use large quantities of blood owing to its scarcity. Since, however, eggs from one female only were used in any given experiment, this was found no great difficulty, since the number of eggs used was very small in each case.

The method used is about as follows: Equal parts of cœlomic fluid and sea-water made solution No. 1. To half of No. 1 was added a like quantity of sea-water to make No. 2. Thus a series of half dilutions was made. One half of the last member in the series was discarded in order that all numbers would contain the same quantity of solution. Uninseminated eggs were placed in each solution—one drop of an egg suspension to each. Likewise a drop of uninseminated eggs was placed in normal sea-water equal in amount to that of mixture of cœlomic fluid and sea-water. The eggs in all dishes were then inseminated with the same amount of sperm from one male. In general, inseminations were made first in 100 per cent. and in 50 per cent. blood. Unless these gave high percentages of inhibition, I made no further dilutions.

The appended summary (Table I.) gives the results of six experiments made in 1919:

TABLE I.

THE INHIBITORY EFFECT OF SPECIFIC BLOOD ON FERTILIZATION OF THE EGG OF *Echinarachnius parma* AS REVEALED BY THE PER CENT. OF CLEAVAGE IN VARIOUS CONCENTRATIONS OF BLOOD IN SEA-WATER IN 6 EXPERIMENTS OF 1919.

No.	Per Cent. of Blood in Sea-water.	Per Cent. of Cleavage.					
		Exp. 1.	Exp. 2.	Exp. 3.	Exp. 4.	Exp. 5.	Exp. 6.
1.....	100	0	0	0	0	0	0
2.....	50	0	0	0	0	0	0
3.....	25	0	0	0	3	6	0
4.....	12.5	0	1	0	8	33	0
5.....	6.25	0	12	16	14	50	0
6.....	3.125	49	40	47	33	81	0
7.....	1.5625	89	64	78	90	90	0
8.....	0 (control)	99	100	98	98	99	36

It is thus seen that eggs of high fertilization capacity fail to fertilize if inseminated in the presence of certain concentrations of blood. Not all eggs give results comparable to those in the table. Thus during May, 1921, several samples of blood tested showed very weak inhibitory power. In essentials, however, the results are quite comparable to those obtained by Lillie in his study of fertilization in *Arbacia.* Moreover, Lillie's interpretation of the mode of action of the blood inhibitor is sustained by this work on the egg of *Echinarachnius,* as will now be shown.

Sperm of *Arbacia* readily agglutinate in mixtures of *Arbacia* egg-water and blood as though the blood were absent. Lillie thus concluded from this that the blood does not block the reaction between the sperm-agglutinating substance (fertilizin) and the sperm; the block comes between fertilizin and substances in the egg. But it is at once apparent to the reader that this is not wholly conclusive: the substance in blood that inhibits fertilization may well do so by some action on the surface of the egg rendering sperm attachment and penetration impossible. Thus it might well be that sperm in the presence of blood and egg-water rich with sperm agglutinin of high power agglutinate; but in ordinary insemination this amount of agglutinin is not present, nor is the insemination made as heavy as the sperm suspensions must be to detect the presence of agglutinin. In the inseminations usually employed for fertilizing eggs agglutination of spermatozoa does not occur; instead, the spermatozoa stick to the egg. As a matter of fact, sperm likewise stick to *Echinarachnius* eggs inseminated in blood. The failure of such eggs to fertilize can not, therefore, be attributed to the effect of blood in blocking the agglutination of sperm to the egg.

At first I considered this result as due to the poor quality of the sperm; that it was not so much an inhibition by blood as a failure of fertilization. Subsequently it was found repeatedly that on inseminating in the presence of blood spermatozoa are attached to the eggs. Thus we have evidence to support the postulate offered by Lillie as to the mode of action of blood inhibitor. This is brought out again in the next group of experiments.

II.

It has been shown (Just, '19) that the fertilization of eggs of *Echinarachnius* by *Arbacia* sperm is greatly facilitated by the use of alkali or by heavy insemination. Though giving a lower per cent. of cleavage than alkali, heavy insemination was for several reasons the method adopted in the experiments made to determine the effect of *Echinarachnius* blood on fertilization by *Arbacia* sperm. That this cross is inhibited by blood was suggested in the earlier work. The experiments now cited indicate that this is true. I cite four experiments made in June and in August, 1920.

Uninseminated eggs of *Echinarachnius* are washed in sea-water and allowed to settle. Five drops of this suspension is distributed equally among five dishes as follows: *Lot A,* uninseminated in sea-water; *Lot B,* inseminated in sea-water with *Echinarachnius* sperm; *Lot C,* inseminated in 3 per cent. *Echinarachnius* blood with *Echinarachnius* sperm; *Lot D,* heavily inseminated in 3 per cent. *Echinarachnius* blood with shed *Arbacia* sperm; *Lot E,* heavily inseminated in sea-water with shed *Arbacia* sperm. The results follow:

Lot.	Per Cent. of Cleavage.			
	June 22.	June 24.	August 4.	August 5.
A.........	0	0	0	0
B.........	99	100	78	81
C.........	74	69	54	49
D.........	0	0	0	0
E.........	43	34	21	17

In some cases a concentration of *Echinarachnius* blood that has *no* effect on the fertilization of *Echinarachnius* eggs by its own sperm will not give a single membrane or cleavage with heavy *Arbacia* sperm suspension. Again, shed eggs of *Echinarachnius* are superior to eggs cut out of the ovaries for cross fertilization. Also, after thorough washing, eggs cut out of the ovaries and fertilized with *Arbacia* sperm yield a higher per cent. of cleavage. I interpret these facts as indicating that it is the presence of blood that makes cross fertilization difficult. Blood thus acts in cross fertilization as it does in straight fertilization; the differences are quantitative only. For *Arbacia* sperm enter *Echinarachnius* eggs in the presence of blood, but they set up no reaction. In my 1917 series of *Echinarachnius* eggs, heavily inseminated with *Arbacia* sperm, this is clearly shown in the sectioned material. Moreover, these eggs are viable; though literally studded with *Arbacia* sperm, they are capable up to twenty-four hours later on insemination with *Echinarachnius* sperm of giving development of a high degree of normality.

It should be noted that in these experiments clean shed sperm of *Arbacia* was used. If the sperm be obtained from the testes admixed with blood, the per cent. of cross fertilization is reduced. This has been repeatedly observed. In some cases, indeed, the shed sperm may give around 30 per cent. fertilization and the sperm from the testes no fertilization. I believe that this is due to the toxicity of *Arbacia* blood. This toxicity is well known from Lillie's observations. I have likewise mentioned elsewhere that *Arbacia* blood is markedly toxic for *Nereis* eggs.

Similarly, mixtures of shed *Arbacia* sperm and shed *Echinarachnius* sperm exhibit no antagonism; eggs of either form or of both dropped into such sperm mixtures fertilize. With mixtures of sperm cut from the testes the results are different, for such mixtures cut down the per cent. of cleavage. In one experiment made with mixtures of shed *Arbacia* sperm mixed with shed *Nereis* sperm there was no sperm antagonism, since eggs of each form developed upon inseminations from the mixture.

These, then, are the results of inseminating eggs of *Echinarachnius* in its body fluid or own blood.

We may conclude: (1) Blood blocks straight fertilization. (2) Blood blocks cross fertilization. (3) Blood blocks both straight and cross fertilization after the spermatozoa stick to the eggs or enter them and not by preventing the attachment of spermatozoa to the eggs.

These conclusions admit of certain suggestions concerning the nature of specificity in the fertilization reaction. We may discuss these briefly.

III.

The block to cross fertilization is cortical. As Lillie says: "The various methods used to induce hybrid fertilization—staling of eggs, high concentration of sperm, use of alkalies or other chemicals—have therefore this one feature in common, that they destroy the chemical or physical integrity of the cortex of the egg" (Lillie, '19, page 219). Specificity in fertilization thus manifests itself in the cortex of the egg.

But specificity in fertilization is not absolute, but relative. This fact would seem to indicate that the results of straight and of cross fertilization are due to quantitative, not qualitative, differences in the cortical response to insemination; species sperm more readily than foreign sperm overcome the same resistance to fertilization set up by some cortical substance or condition. The question, therefore, comes down to this: What in the cortex is responsible for the block to fertilization, whether by species or foreign sperm?

In the first place, most methods used to induce cross fertilization in echinids hasten the loss of fertilizin. Thus staling is an easy method for the removal of fertilizin. Eggs allowed to stand or repeatedly washed lose their fertilizin content. Washing the eggs rapidly with dilute sea-water brings about a loss of fertilizin. Dense sperm suspensions rapidly bind available fertilizin. I venture the opinion that heat hastens the loss of fertilizin also.

If, now, we postulate that specificity in fertilization is wholly due to the presence of fertilizin, then must we also take the next step, namely, that cross fertilization is most successful when the fertilizin is reduced? That is, fertilizin is necessary for straight fertilization, but a block to cross fertilization; certain kinds of artificial parthenogenesis (heat, for example, on *Nereis* egg) depend upon the presence of the fertilizin in maximum concentration; certain eggs lose their capacity for fertilization by species sperm very rapidly (*Platynereis*); but with foreign sperm the case is otherwise—it can fertilize after an egg is no longer capable of response to artificial stimulus or that of species germ. But might not specificity in fertilization be accounted for in part on the basis of the data presented in this paper? This would mean at least with the knowledge at hand that specificity in fertilization is due in part to the blood, since the presence of blood blocks fertilization by species or foreign sperm.

When species sperm comes in contact with an egg, it gains entrance and fertilizes against the blood present. The greater the amount of blood, the more difficult the fertilization. Indeed, the blood may actually inhibit fertilization in every egg. Therefore, dense sperm suspensions must be employed for fertilization in the presence of blood rich in inhibitor. The blood inhibitor acts by binding the fertilizin so that the fertilizin can not react with the egg receptors. Heavy insemination insures fertilization perhaps by increasing the chances of some spermatozoa locating fertilizin free of blood inhibitor. Or in heavy insemination the onslaught of numerous sperm brings it about that the fertilizin shakes free the inhibitor.

The blood slowly leaves the egg as it lies in sea-water. But the fertilizin also goes. Hence while the egg is losing inhibitor it is also losing fertilizing power. The blood is perhaps never an irremovable block to species sperm; however, though present in but a trace, it serves to block foreign sperm. In staling, therefore, what results is not only loss of fertilizin, but also loss of blood. The loss of blood makes possible cross fertilization.

What is true of staling is doubtless true of other methods for obtaining cross fertilization—heat, use of alkali, and of dilute sea-water; they serve to remove the blood block. The fertilizin remains albeit in reduced quantity. Whenever an egg is capable of fertilization it possesses the fertilizable substance. And it is safe to assume that an egg that will not respond to its own sperm will not cross fertilize.

From this point of view, then, fertilizin is not the only factor in specificity. It is specific since it engages species sperm against the inhibition of blood. But the blood is an aid to specificity, since it blocks all sperm, species sperm least of all.

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THE FERTILIZATION–REACTION IN *ECHINARACHNIUS PARMA.* VIII.

FERTILIZATION IN DILUTE SEA-WATER.

E. E. JUST,[1]

ROSENWALD FELLOW IN BIOLOGY, NATIONAL RESEARCH COUNCIL.

The writer has shown,[2] by means of experiments made in 1920 at the Marine Biological Laboratory, Woods Hole, Mass., that a given dilution of sea-water with tap water which leaves the uninseminated egg of *Echinarachnius parma* intact is rapidly injurious to the inseminated egg during its process of membrane separation. After membrane separation the egg is likewise resistant to the action of the dilute sea-water. Certain experiments were also made during the same season to learn if it is possible to procure fertilization and development in diluted sea-water to which the inseminated egg during membrane separation is susceptible. These experiments were repeated during May and June of the 1921 season at the laboratory with essentially the same results.

In experiments made to discover if in a given dilution of sea-water, which is injurious to the inseminated egg of *Echinarachnius* during the period of membrane separation, it is possible to fertilize the egg, it was soon apparent that cleavage and normal gastrulation are not possible in a dilution which permits membrane separation. Thus during the period June 21 to June 28, 1921, inclusive, eggs from twenty-four females were inseminated in normal sea-water and in sea-water of varying dilutions. In all cases due precautions were taken as to the bulk of eggs, quantity of solution, and density of the sperm suspensions used in order that as far as possible conditions be made uniform. The results obtained with 95 per cent. sea-water (95 parts sea-water plus 5 parts tap water) and the dilutions ranging from this to 80 per cent. sea-water (80 parts sea-water plus 20 parts tap water) may be summarized briefly as follows: In 95 per cent. sea-water eggs fertilize normally as revealed by the per cent. of membranes formed. They cleave and gastrulate in this dilution. In most cases the eggs are scarcely to be distinguished from eggs inseminated in normal sea-water. In 90 per cent. sea-water membranes are normal and cleavage fairly normally; but these eggs form about 10 per cent. exogastrulæ. In 85 per cent. sea-water the per cent. of membranes is close to normal—95 per cent. or slightly more. The cleavages show some abnormalities and the per cent. of exogastrulæ is greater than in 90 per cent. sea-water. In 80 per cent. sea-water the per cent. of cleavage falls and the per cent. of exogastrulæ increases.

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[1] Zoölogical Laboratory, Howard University.

[2] Am. Jour. Phys., 1922, 61, 516.

The experiments indicated that the dilution made up with 75 parts sea-water plus 25 parts tap water is the lowest which permits cleavage. In this the gastrulæ are very abnormal. We may, therefore, turn our attention to the experiments made with this and greater dilutions of sea-water. Eggs inseminated in dilutions greater than those that permit cleavage separate membranes. Even in the dilution made up of equal parts tap water and sea-water some membranes separate, though these are often hard to see. Eggs, therefore, will respond to insemination with at least abortive cortical changes, though they can not cleave. I cite now one experiment which was made—as the majority of these experiments—on the eggs of three females.

June 21, 3:30 P.M. Six series of dilute sea-water, each consisting of 6 members, as follows:

No.	1.	2.	3.	4.	5.	6.
Parts sea water	50	55	60	65	70	75
Parts tap-water	50	45	40	35	30	25

Equal portions of uninseminated eggs from each of 3 females (*A, B,* and *C*) put in each dilution of sea-water to make Series I*A*, I*B*, and I*C*, II*A*, II*B*, and II*C*.

Series I*A*, I*B*, and I*C* uninseminated.

Series II*A*, II*B*, and II*C* inseminated with same quantity of sperm from one male. Inseminations in each series 5–25 seconds after eggs in dilute sea-water.

Controls: uninseminated eggs in normal sea-water; inseminated eggs in normal sea-water.

The eggs were examined at intervals. The next day, 7:50 A.M., the uninseminated eggs (Series I*A*, I*B*, and I*C*) showed an average of less than 10 per cent. cytolysis. The history of Series II. is given in the table below (Table I.):

TABLE I.

PER CENT. OF MEMBRANES AND OF CLEAVAGE IN EGGS OF *ECHINARACHNIUS* IN-IN SEA-WATER OF VARYING DILUTION.

No.	Amount of Sea-water in 100 Parts of Sea-water plus Tap Water.	Per Cent. of Membranes in Eggs from the 3 Females.			Per Cent. of Cytolysis Two Hours after Insemination.			Per Cent. of Cleavage 5 Hours after Insemination.		
		A.	*B.*	*C.*	*A.*	*B.*	*C.*	*A.*	*B.*	*C.*
1	50	0	2	1	18	4	9	0	0	0
2	55	0	2	0	4	2	1	0	0	0
3	60	7	7	7	0	3	0	0	0	0
4	65	5	22	20	0	1	0	0	0	0
5	70	14	63	47	0	3	0	0	0	0
6	75	60	95	87	0	2	0	5	16	4
Uninseminated control	100	0	0	0	0	0.1	0	0	0	0
Inseminated control	100	100	98	100	0	0.5	0	100	97	100

This experiment indicates that eggs inseminated in dilutions of sea-water may separate membranes, though they do not cleave. It would, therefore, be erroneous to assert—if membrane separation be the criterion for fertilization—that eggs that do not cleave have not been fertilized. Rather the failure to cleave is due to the action of the dilute sea-water in interfering with the cleavage mechanism—particularly with the activity of the hyaline plasma layer. There is no question here of "partial fertilization"; it is wholly a question of incomplete cleavage. This is important for the experiments that we may now consider.

In the experiments now to be considered it was repeatedly found that eggs may be inseminated in a dilution that is destructive to the egg which is inseminated in sea-water and exposed to this dilution during membrane separation. The criterion of this destructive action is the differential cytolysis of uninseminated and inseminated eggs before, during, and after membrane separation.

With cleavage, however, the case is quite different. That is, the per cent. of cleavage of eggs inseminated in a given dilution is no higher than that of eggs inseminated in sea-water and transferred to the dilution before, during, or after membrane separation. There might be a slight indication that eggs exposed during membrane separation show a lower per cent. of cleavage. I believe, however, that my figures on this point are not decisive.

I give now one experiment to show the per cent. of cytolysis in eggs inseminated in dilutions of sea-water as compared with that of eggs inseminated in sea-water and placed in the dilutions before, during, and after membrane separation.

June 27, 11:25 A.M. Following dilutions prepared:

No.	1.	2.	3.	4.	5.	6.
Parts tap-water	50	45	40	35	30	25
Parts sea-water	50	55	60	65	70	75

Five dishes for each of the six dilutions containing 10 c.c. each. Thus 5 series of 6 numbers each.

Drops of uninseminated eggs from one female added to dilutions as follows:

Series 1: 1 drop of eggs in each dilution.

Series 2: 1 drop of eggs in each dilution; inseminated immediately.

Series 3: Drop of eggs to each dilution 15 seconds after insemination in sea-water.

Series 4: Drop of eggs to each dilution during the period of membrane separation 35 seconds after insemination in sea-water.

Series 5: Drop of eggs to each dilution two minutes after insemination in sea-water.

Controls: Uninseminated eggs in normal sea-water; inseminated eggs in normal sea-water.

The results of this experiment follow:

12 M.

Series 1: Less than 1 per cent. cytolysis.

Series 2: As in Series 1.

Series 3:
(12:00 M.)

No.	Dilution (Parts of Sea-water in 100 Parts of Solution.	Per Cent. of Eggs Intact.	Per Cent. of Eggs Cytolyzed.
1	50	79	21
2	55	77	23
3	60	89	11
4	65	98	2
5	70	97	3
6	75	100	0

Series 4:
(1:00 P.M.)

No.	Dilution (Parts of Sea-water in 100 Parts of Solution.	Per Cent. of Eggs Intact.	Per Cent. of Eggs Cytolyzed.
1	50	50	50
2	55	47	43
3	60	85	15
4	65	80	20
5	70	90	10
6	75	97	3

Series 5:
(1:30 P.M.)

No.	Dilution (Parts of Sea-water in 100 Parts of Solution.	Per Cent. of Eggs Intact.	Per Cent. of Eggs Cytolyzed.
1	50	69	31
2	55	83	17
3	60	86	14
4	65	97	3
5	70	99	1
6	75	98	2

Controls: Uninseminated in sea-water—1 per cent. cytolysis.
Inseminated in sea-water 99 + cleavage.

It is clear from this experiment that eggs exposed to a dilution of sea-water during membrane separation cytolyze in slightly higher per cent. than eggs inseminated in the dilution. Similarly, eggs inseminated in normal sea-water and exposed before or after membrane separation cytolyze in slightly lower per cent. than eggs exposed during membrane separation. The cleavage per cent. of *surviving* eggs is about the same.

If we hold that membrane separation is a criterion of fertilization, then eggs inseminated in a given dilution of sea-water are fertilized whether they cleave or not. This admittedly is not a strong case, but it gives some support to the position that the fertilization reaction is practically instantaneous. It is not fertilization that is checked by the action of dilute sea-water, but the reactions which set up by fertilization lead to cell division.

REACTIONS OF CELL-BODIES AND PSEUDOPODIAL FRAGMENTS OF *DIFFLUGIA*.[1]

WM. A. KEPNER AND B. D. REYNOLDS,
University of Virginia.

Introduction.

The purpose of this paper is to record a number of observations which were prompted by an incident occurring on October 7, 1919, while one of us (Kepner) was watching a *Difflugia pyriformis* Ehrenberg under the compound microscope. The animal suddenly retracted an unusually long pseudopod, and in doing so its distal end was torn off. In a short time the fragment began to show amœboid movement; meanwhile the animal sent out other pseudopods, and within a few minutes the two masses of protoplasm made contact and fusion took place. This naturally called to mind the work which had been done by others on regeneration and restitution; therefore a series of experiments were undertaken in order to find out to what extent, if any, this phenomenon was exhibited in *Difflugia*.

It is generally known that many animals possess conspicuous powers of regeneration. For example: snails are able to regenerate tentacles; earthworms, flatworms, and polyps may have their bodies severed and each fragment be able to restore the parts that it lacks to complete a body. Verworn (1892) observed that severed fragments of *Orbitolites* would occasionally adhere to the ends of parental pseudopods. Jensen (1896) pointed out that *Orbitolites* and *Amphistegina* (belonging to the order *Foramifera*) would sometimes recover fragments belonging to themselves and make them again a part of their bodies. Wilson (1907, '11) has shown that the tissues of sponges broken up or macerated by passage through the meshes of bolting cloth, so that the component cells are separated and distributed in water, will reassemble and form new individuals.

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[1] This is the first of a series of contributions on this subject. More extensive observations and experiments are now being conducted by the junior author with other genera of Rhizopods, as well as with the genus *Difflugia*.

The conditions of life under which the above animals live impose a risk of the body parts. *Difflugia* is protected, to a certain extent, from external environments by its shell, and perhaps also by the formation of a plug of mucus about the mouth of its shell when unduly stimulated. Nevertheless, in sending out long, slender pseudopods, it, too, runs a risk of losing some of its protoplasm, for, as recorded by Verworn (1890), when the projecting pseudopods are strongly stimulated, they are frequently jerked back so suddenly as to whip off their ends.

The Organisms.

Various species of *Difflugia* were used in these experiments, all of which gave similar results. However, it was found that *D. acuminata, D. corona, D. pyriformis, D. spiralis,* and *D. vulgaris* were more favorable for study because of the nature of their pseudopodial formation, and also because they commonly drag themselves after their advancing pseudopods with the shell in a reclining position, so that the mouth of the shell and the bases of the pseudopods can be seen easily. This feature also affords a better opportunity for cutting off pseudopods without danger of striking the shell.

Methods.

(*a*) *Collecting.*

The animals were collected in small puddles in a cow pasture near the University of Virginia. These puddles were near a fresh stream and frequently obtain a supply of fresh water, due to rains, and the overflowing of the banks of the stream. In the same puddles other protozoa, fresh-water crustacea, and various algæ are also found. The brown ooze and vegetable matter on the bottom of these little pools were carried to the laboratory in fruit jars, then allowed to stand for several hours, after which the surface of the sediment, along with sufficient water to fill the vessel to a depth of approximately five millimeters, was transferred to Petri dishes. The organisms can be kept in this way for several weeks, provided fresh water is added occasionally. In these experiments no attempt was made to maintain pure line cultures.

(*b*) *Apparatus and Technique.*

Individuals can easily be isolated for study by placing a Petri dish, containing the organisms, under a low-powered binocular, locating the animal desired, and transferring it by means of a narrow-mouthed pipette to a plain slide containing a large drop of spring (or distilled) water. The amputations were made with fine glass needles, which can readily be drawn in a small hot flame. It is essential to use hard elastic glass for this purpose.

At first the operations were made under a binocular, and the slide containing the organism was then transferred immediately to a compound microscope for observation, but later operations were made under the compound microscope, using a 32-mm. objective and a No. 10 eyepiece (B. & L.). The latter method is altogether more satisfactory, since it enables the observer to keep sight of the performance from the very beginning, and also avoids any jarring or shifting of the protoplasms, which might take place while transferring the slide. Practically all of the observations were made under the 16-mm. objective and No. 10 eyepiece. Most of the distances given in this paper were estimated either in terms of the diameter of the field or in terms of the diameters of the shells of the individuals concerned. A later and more satisfactory method is to use a camera lucida and trace the outlines of the experimental objects at definite intervals. By this method exact record can be kept of the relative sizes and positions, as well as the paths followed by each body.

I. Is Reunion of Cell-body and Fragment the Usual Occurrence?

In order to determine this we performed approximately one hundred experiments, the majority of which gave positive results. The few negative cases observed were apparently due to one of the following causes: (*a*) Severe injury to cell with consequent failure on its part to extrude pseudopods, (*b*) separation from fragment by too great a distance, (*c*) evaporation of water before completion of reaction. These results have been repeatedly confirmed by graduate students in this laboratory. Since mucus is involved in the organisms' locomotion, it occurred to us that the separated parts might follow a mucous trail, or even invisible strands of protoplasm, and thus contact would be effected. For this reason, in most cases after a pseudopod was severed the cell-body was moved to a considerable distance, and yet the fragment was recovered. Again the cell-body has been moved in a wide detour around on another side of its fragment, after which it would approach without regard to the course along which it had been pushed. The mouth of the shell has been placed in every angle to the fragment from zero to one hundred and eighty degrees; nevertheless the fragment is usually recovered. To give the details of each experiment would require too much space and necessitate repetition of similar results; therefore, the following two observations have been selected as typical of the usual reaction:[1]

On July 24, 1920, the end of a pseudopod was cut from a *Difflugia vulgaris* by Mr. E. Paylor, of this laboratory, after which the cell-body was immediately moved 500 micra away from it, the mouth of the shell being directed away from the fragment, as shown in Fig. 1—*A*. Within a minute the animal, which had withdrawn into its shell after the operation, began to send out two pseudopods. After these pseudopods had been extended to a distance of about fifty micra, their ends adhered to the glass slide on which the animal was lying. Then by a movement somewhat similar to a hand-spring the body proper was thrown over the extended pseudopods, so that now the mouth of the shell was pointed toward the fragment (Fig. 1—*B*). The animal then began to move in the direction of the fragment, moving in a counter-clockwise curve as indicated by Fig. 1—*C, D, E,* and *F*. This movement was continuous until the fragment was encountered and reappropriated through fusion of the two masses of protoplasm (Fig. 1—*G*).

The second example given to illustrate the phenomenon of fusion is perhaps more interesting, for in this case more than one fragment was involved. In this experiment exact record of the time at which each event took place was made. At 11:38 A.M., June 10, 1920, a small fragment, *c,* Fig. 2, was cut off from a *Difflugia spiralis.* The animal immediately withdrew its extended pseudopods, but not completely within its shell. It then sent out two new pseudopods, and at 11:39 A.M. the ends of both these were cut off, *a* and *b.* The animal was so stimulated by this second operation that it quickly retracted its pseudopods, and in doing so was turned through an angle of ninety degrees, so that now its mouth lay directed at right angles to the fragments, which were lined up in a row. In a short time the animal extended a short pseudopod which curved around alongside the neck of its shell and fused with *a* at 11:40½ A.M. The cell-body was then moved 600 micra away and placed so that the side of its shell was toward the remaining two fragments which had been left in their original positions. By 11:45 A.M. it had advanced again and fused with fragment *c.* The animal was then stimulated with a glass rod, so that it withdrew into its shell again; but by 11:45¾ A.M. it had sent out another pseudopod which fused with fragment *b.* Thus within seven and three quarter minutes from the time the first fragment was cut off the animal had picked up and reappropriated all three fragments, though during the interval it had been stimulated by the observer so violently as to make it contract four times, and at one time it was moved six times its shell's diameter away, through which distance it had to travel in order to get back to its missing parts.

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[1] We realize that the conclusions drawn in this paper would be more convincing if we published our data in extenso, but since this is a preliminary report of observations on a subject which is now being actively investigated with a view to further and more extensive publications, it seems best to present our results here by means of typical experiments and general statements regarding the entire investigation.

It has not been determined exactly what the maximum range of positive response is—*i.e.,* the limit within which fusion always occurs. One positive case was observed in which the cell-body and fragment were separated by one and one half millimeters. But chance wandering might have brought the animal within the positive range. Be this as it may, chance can hardly explain the large percentage of positive observations made. When the two elements were separated by great distances, we encountered a small percentage of negative reactions. However, in every case which we observed where the cell-body and fragment were separated by a distance of 500 micra or less fusion always took place, unless the cell-body was injured. It seems, then, that reunion is the usual thing which takes place, if the missing parts be not too far removed.

II. Is the Phenomenon a Process of Feeding?

From the experiments described in the preceding section the conclusion was drawn that *if a fragment of protoplasm be severed from a Difflugia, it is usually reappropriated.* The question naturally arises: Does the animal react to its fragment as a piece of food, or do the two coalesce? If the fragment is taken up as food, we would expect to see: (*a*) formation of a food vacuole and enlargement at site of ingestion, (*b*) a similar reaction to fragments from closely related forms, and (*c*) no sharp distinction as to whether or not the fragment showed visible signs of life, such as movement.

(*a*) In some of these observations the animals were in such a position at the time contact was made that it was impossible to see what took place on account of the shell, but in the majority of cases the view was unobstructed, so the entire process could be witnessed. The usual reaction is approximately as follows: As the advancing pseudopods of the cell-body near the fragment they usually cease to become attached to the substratum, as in the ordinary process of locomotion; thus movement on the part of the animal is slowed down. If at this time only one pseudopod is extended, smaller secondary ones may be sent out near its base or from the mouth of the shell. The primary pseudopod may make direct contact with the fragment, or it may form a small cup into which the fragment is fitted, or a niche may be formed by the extrusion of one or more secondary pseudopods, and contact is made in the niche thus formed. If there are two or more primary pseudopods, they may encircle the fragment collectively, or only one of them may take part in the reaction. When contact is made there is ordinarily a disturbance of the protoplasmic surfaces, which varies from a mild local shock to a violent contortion involving the entire (visible) protoplasm. When the "contact shock" is very great it is impossible to observe what becomes of the fragment, but when it is slight the granular ectoplasm constituting the fragment can be seen streaming into the pseudopod and becoming disseminated, as though the fragment were a lateral branch which the pseudopod was withdrawing. There is no sign of incoherency or enlargement at this place (see Fig. 5—*A, d*).

(*b*) The reaction toward foreign fragments is different, as will be brought out later.

(*c*) On many occasions it was observed that after fragments had been separated from their cell-bodies for a long time they would cease to move, become rounded off, and take on an opaque granular appearance. The time required to bring about this change depends on several conditions, particularly on the size of the fragment; a large fragment retaining its power of movement longer, other things being equal. Though it has not been determined experimentally, it is reasonable to presume that the physiological condition of such a fragment is changed as well as its appearance; yet its molecular composition can hardly be so altered as to change its food value. Though over twenty-five observations have been made on the reaction between *Difflugia* and its fragment, which had assumed a granular, opaque, spherical condition, not a single instance was found in which the animal picked up such a fragment. The following example is illustrative of the reactions obtained:

On June 10, 1920, at 11:47 A.M., a large pseudopod was cut from a *Difflugia spiralis* and the cell-body was then moved out of the field. The fragment moved about for fifteen minutes, then gradually ceased and assumed a spherical shape. By 12:15 half of the bulk constituting this mass of protoplasm showed disintegration to such an extent that it had changed from the spherical condition and was scattered, in an uneven manner, around the portion which still retained its rotundity. At this time the cell-body was brought up and the mouth of its shell placed in contact with the fragment. The animal soon extended two pseudopods which came in contact with the fragment in the manner indicated in Fig. 3—*A*. For two minutes the animal remained practically passive, then it began to move forward, taking with it the portion of the fragment which was still in a spherical condition. This was held by a secondary pseudopod which had formed an imperfect cup around it (Fig. 3—*B*), the disintegrated portion being left behind. Movement was continued in the same direction until the animal had traveled twice the distance of the diameter of its shell. But at 12:22 P.M. it rejected the portion of the fragment which it held and reversed its course, leaving the fragment behind.

From our observations, then, it seems evident that severed fragments are not taken up by the cell-body as food, but that the separated bits of protoplasm recombine with the original mass and take up their function as though nothing had happened, provided disintegration processes have not set in before contact is made.

III. Does Fusion Take Place at Any Point Along the Pseudopod?

A *Difflugia* progresses by extending pseudopods (sometimes they are very long and slender), the ends of which become attached to the substratum. With this in mind, one would naturally expect that the end of an advancing pseudopod would first make contact with a fragment which the animal was approaching. On the contrary, this has never been observed. In the last section attention was called to the fact that when nearing a fragment the ends cease to become attached. At this stage they usually wave around in the medium, and further progression is made either by this waving motion, or else by smaller pseudopods near the mouth of the shell. Though not all cases were quite so clear cut, the following example illustrates the principal region of a pseudopod involved:

On October 7, 1919, a *Difflugia pyriformis* was observed extending a large pseudopod toward its fragment *a* (Fig. 4—*A*). In the meantime the fragment was moving in the direction shown by *e, f,* and *g* (Fig. 4). After its end had passed well beyond the fragment, the pseudopod was bent toward the fragment until its side had come in contact with the rear end of the moving ectoplasmic body. When this contact was made, the animal threw out an intimately fitting cup around the posterior lobe of the fragment (Fig. 4—*B, g,* and *g′*). Immediately the protoplasm, which had been detached, flowed back into the pseudopod, without causing any perceptible increase in its diameter.

In all cases which we observed fusion took place along an extended mid-region, neither at the tips nor at the bases of pseudopods.

IV. Does the Fragment Take an Active Part in Restitution?

If fusion between a cell-body and its fragment is true restitution as has been claimed, one should not be surprised to see the fragment taking an active part in the process. With this in view, especial attention was given to the movements of fragments. Without some object to mark the original positions, it is very easy to confuse distances traveled by each body when two are approaching each other. Therefore, when observations were made without the use of a camera lucida to plot the positions, care was taken to avoid falling into this error. Whenever fusion took place, the protoplasm constituting the fragment seemed to flow into, and commingle with, the protoplasm of the cell-body. But it was not always possible to observe an appreciable movement on the part of a fragment toward its cell-body before contact was made. However, on many occasions this could be seen. The two following accounts are typical of the more striking reactions of this sort:

On November 28, 1919, a fragment was cut from a *Difflugia spiralis,* after which the cell-body was moved 1,500 micra distant. Immediately after separation the fragment had the shape of an elongated ovoid, then its contour changed continually for the next thirteen minutes (Fig. 5—3, 4, 5, 6, 7, 8, 9, etc.). Each figure represents the number of minutes that had elapsed since the operation took place. During the first twelve minutes its movement seemed to be indefinite, as indicated in the diagrams, but by this time the cell-body had advanced until the end of its nearest pseudopod was within 150 micra of the fragment. From this time there was a decided movement on the part of the fragment in the direction of the cell-body. As soon as the fragment had come within reach of the long pseudopod, which the cell-body was extruding, movement by the latter slowed down, and the distal end of the pseudopod was lifted. Under this the fragment passed. The fragment continued the approach, and two minutes from the time it had come within the radius of the animal's pseudopod it had traveled about twenty micra and reunited with its cell-body (Fig. 5—*A*, *a*, *b*, *c*, and *d*).

On November 8, 1919, a large fragment was cut from a pseudopod at 10:44 A.M. This soon became greatly contorted, and from its tangled mass two amœboid fragments emerged. The water became shallow soon after the operation and it was necessary to add more, which separated the fragments a considerable distance from the cell-body. At 11:16 A.M. the fragments were placed in the vicinity of the cell-body again (Fig. 6—*A, a, b*). At 11:23 A.M. fragment *a* had shifted its axis so that it now lay directed toward the mouth of the animal's shell. Fragment *b,* during the same period, traveled to position *x*. The cell-body was now pushed up to the relative position shown at *B* without disturbing the fragment. The anterior tip of fragment *b* was immediately shifted so that it was now directed toward the new position of the shell's mouth. The entire fragment had advanced to position *y* by 11:27 A.M. At this time fresh water was added to the slide, and in doing so the fragments were lost.

That such enucleated fragments show a positive response to stimulus, whatever it be, seems unquestionable. The importance of this is, perhaps, emphasized by the fact that all fragments involved in our observations were not simply enucleated bodies of protoplasm, for endoplasm did not constitute a portion of any of them. They were, therefore, *enucleated ectoplasmic fragments.*

V. Will Two Enucleated Fragments Fuse with Each Other?

Having observed the readiness with which fusion took place between *Difflugia* and its ectoplasmic fragment, the question suggested itself—*Is there a tendency between two pieces of protoplasm to fuse when no restitution of a cell would result from such a fusion?* Not a sufficient number of experiments has been performed to warrant an emphatic statement in regard to this point. But at least twenty observations have been made in which two or more fragments from the same organism were involved. In these experiments the fragments were only ectoplasmic in composition and varied in size from five to twenty micra in diameter. The results of these experiments can be illustrated by the following example:

A large and a small fragment were cut from a *Difflugia spiralis,* after which the animal was removed from the slide. The fragments were separated by the distance of about fifty micra, and for two minutes they moved around quite actively, but no progress was made toward uniting. They were then placed in contact, by using a glass needle, so that now they were in the positions shown in Fig. 7—*m*. This contact was a very close one, the adjoining surfaces being very extensive and the line of demarcation quite indistinct, yet discernible. Ten minutes later the activity of the fragments had begun to cease and they showed signs of rounding up into spherical bodies, each piece into its respective sphere (Fig. 7—*n*). After ten more minutes the spherical condition had been attained and the pieces showed only a small point of contact (Fig. 7—*o*).

Thus, after having been placed in direct contact, there was a failure on the part of these two fragments of ectoplasm to unite—though they had come from the same cell. It seems, then, from our observations, that enucleated ectoplasms do not fuse with each other.

VI. Will *Difflugia* Fuse with Pseudopodial Fragments Belonging to an Individual of a Different Species?

(*a*) *As Food.* (*b*) *By Protoplasmic Union.*

The criteria for determining whether or not the fragments serve as food have been set forth in a previous part of this paper. However, since the reaction of an individual toward fragments belonging to closely related forms was proposed as one method for determining this, these experiments were designed to shed some light on this subject. Furthermore, it occurred to us that in such organisms an individual might fuse with foreign fragments in the same manner described for cell-bodies and their own fragments. The results obtained were such that it will not be necessary to make a distinction between (*a*) and (*b*) in discussing the reactions. The method which we employed was to take two *Difflugia* of different species and place them near each other in a drop of water, and as soon as one of the animals had extruded a long pseudopod it was cut off and the animal then removed from the drop, leaving its fragment and the other individual behind. Attempts were made in this way to cross the protoplasm of all the various species of *Difflugia* previously mentioned, viz.: *D. acumi-*

nata, D. corona, D. pyriformis, D. spiralis, and *D. vulgaris.* While only two or three observations were made on each combination, the results were all negative. Therefore, we feel a degree of assurance in asserting that cross-fusions do not normally take place. The following is one of the most striking observations made on this type of reaction:

On June 10, 1920, a fragment was cut from a *D. spiralis* (animal *A*). This animal was then moved from the field, and a *D. vulgaris* (animal *B*) was brought into contact with the fragment. This contact remained for three minutes, after which animal *B* left the fragment and moved from it to a distance of 200 micra; but within two minutes it had turned around, retraced its course, and made contact with the fragment again. This time such close contact was made that it appeared as though fusion had resulted. Such intimate relations continued for five minutes, then another separation, animal *B* leaving the fragment for the second time. After it had traveled about 300 micra it again turned and came back to the fragment of foreign ectoplasm, making a third contact in about three minutes. This time the two objects did not adhere so closely as on previous occasions. Three minutes later animal *B* moved away from the fragment again, this time remaining away four minutes before returning to make the fourth contact. On this occasion the animal made only slight contact with the fragment, moving off again after a few seconds. When animal *B* had traveled approximately 100 micra, it reversed its course and seemed about to approach the fragment another time. However, before reaching it the animal reversed its course and did not return any more. During the whole procedure (an interval of twenty-six minutes) there was no positive reaction on the part of the fragment of *D. spiralis* toward the *D. vulgaris.*

While it is possible that by using different media different reactions may be obtained, it seems that under conditions comparable to their normal habitat one species of *Difflugia* will not assimilate protoplasm belonging to another species.

VII. Will Individuals Fuse with Protoplasm from Other Individuals of the Same Species?

Being unable to get cross-fusions between different species, we next sought to determine whether or not one individual would fuse with a pseudopodial fragment from another individual of the same species. In order to make conditions similar to those under which organisms have been repeatedly observed to pick up their own fragments, a portion of the protoplasm was removed from the animal concerned before placing a fragment from another individual near it. The following reaction is an example of what commonly happens when dealing with wild cultures:

Two specimens of *D. spiralis* (*A* and *B*) were placed in the same drop of water, and a fragment was cut from animal *A* at 10:24 A.M., July 14, 1921. This fragment was carried out of the water as it clung to the glass filament with which the cutting was done. Animal *B* was now dragged up to the position shown in drawing (Fig. 8). At 10:29 A.M. fragment *c* was cut from animal *B* without disturbing the position of either cell; the operation did not even cause animal *A* to withdraw its large pseudopod. Specimen *B* was immediately carried from the drop. Animal *A* turned away from the fragment to position *A′* and moved off, but a little later it was moved back to less than half its shell's length from the fragment, only to move away again. For the third time animal *A* was placed near the fragment of foreign ectoplasm, and though one of animal *A*'s pseudopods passed directly over it, there was no reaction either on the part of animal *A* or the fragment from animal *B*.

Other reactions have been observed in which individuals did fuse with fragments from animals of the same species. However, in all reactions of this sort the organisms were obtained from the same culture. There are two possible explanations for this: (*a*) *The individuals were closely related by having a recent common ancestor.* (*b*) *By living in the same surroundings, the environmental influences have acted upon both organisms in such a way as to cause an identical physiological state.* This is left as an open question, upon which further work is being done.

Discussion.

Verworn (1892) cut off pseudopods from *Orbitolites* and observed that after the severed masses had rounded up into little droplets they would adhere to the *ends* of pseudopods extended by their cell-bodies. Soon thereafter the masses of severed protoplasm would bend at the points of contact and the contents would flow up the pseudopods. Jensen (1896) pointed out that in *Orbitolites* and *Amphistegina* chance crossing of pseudopods of different specimens may cause instantaneous shattering of the protoplasm concerned, and that the organisms would occasionally pick up the fragments belonging to themselves and make them again a part of their bodies. He was also able to get the same results by cutting off fragments of pseudopods. Furthermore, he observed that one species would react negatively toward fragments from other species. Later, in 1901, he made some further experiments on *Orbitolites* in which he obtained practically the same results. In the last paper he indicates that there is some response on the part of the fragments toward restitution. He calls attention to the fact that tendency toward restitution is stronger just before the severed fragments lose their power of movement. This does not correspond to our findings, and since he does not describe his experiments in detail, it is difficult to know whether or not they have much in common with those made by us.

There are many unicellular animals that may be cut into two parts and each part display vital phenomena. In the uninucleated forms the part, or parts, lacking a nucleus probably carry on only katabolism or destructive processes. In multinucleated protozoa we often find each mutilated fragment becoming a new individual—as, for example: when a *Stentor, Actinosphærium,* or *Arcella* is fragmented. Wilson (1907, '11) separated the cells of a sponge's body and found that these cells would become organized again into a new sponge. But this was restitution through segregation and reorganization of parts of a multicellular organism. In *Difflugia* we have the parts of a cell fusing to retain its former completeness. In this relation it is impressive to observe how definitely ectoplasmic fragments respond, in some cases, to their cell-bodies. As the cell shifts its position the fragment changes its course correspondingly.

Chemotaxis of some sort is probably involved in the attraction shown between cell-bodies and their fragments, but that point has not been determined. Neither mucous trails nor invisible strands of protoplasm are necessary for the protoplasmic reunion to take place, for care has been taken to place the animals in such positions that this could be determined. Some substance may emanate from each *Difflugia,* as products of its individual metabolism, which furnishes the required stimulus.

An interesting feature of the reaction between an ectoplasmic fragment and its cell-body is presented by the fact that fusion has never been observed to take place at the tip of an advancing pseudopod, nor at its base, but always along an extended mid-region. This suggests that the ends of such pseudopods must be different from their sides. This, if well founded, carries the analysis of pseudopodial formation a bit beyond that presented by Hyman (1917), who showed that the younger pseudopods of an amœba have a different metabolic gradient from the older ones.

The ability of enucleated fragments to react to stimuli has been observed by others. Hofer (1889) showed that fragments of amœbæ lived for fourteen days; their movements were carried on, though somewhat modified. Verworn (1889) found that enucleated fragments of *Amœba, Difflugia, Lachrymaria, Polystomella,* and *Thalassicolla* live a long time and perform normal movements and normal reactions to stimuli. Minchin (p. 210) makes this statement: "Non-nucleated fragments may continue to live for a certain time; in the case of amœba such fragments may emit pseudopodia, the contractile vacuole continues to pulsate, and acts of ingestion or digestion of food that have begun may continue; but the power of initiating the capture and digestion of food ceases, consequently, all growth is at end, and sooner or later all non-nucleate bodies die off." Lynch (1919) observed that enucleated fragments of an amœba may move, respire, digest, respond to stimuli, and exhibit any activity which is dependent solely upon katabolic or destructive processes of protoplasm. The group of phenomena which they never show constitutes such processes as growth, regeneration, and division. Willis (1916), on the other hand, records that parts without a nucleus do not react as well as nucleated portions—for example: they do not orient with reference to a beam of light. Finally, Mast and Root (1916) say that "the fact that enucleated parts of amœba do not respond at all or respond in a haphazard fashion indicates, as Hofer ('90) concludes, that the nucleus acts as a regulatory center." Hofer, Willis, and Mast and Root have found, therefore, that enucleated fragments of protozoa do not respond normally, while Verworn and Lynch find that katabolic activity is normal on the part of these enucleated fragments of protoplasm. In *Difflugia* we have found a response that is remarkably well regulated and very delicate. This is of especial interest because the fragments which we were dealing with were only ectoplasmic in composition.

We wish to avoid the implication that the regulated movements of these ectoplasmic fragments were effected without nuclear control, for the absence of nuclei in them does not preclude nuclear influence. Regulatory substances of nuclear origin might have been present in the ectoplasmic fragments at the time they were severed from the cell-body. For, as Minchin (p. 65) states, "in many protozoa, especially amongst the Sarcodina, as, for example, *Arcella, Difflugia,* and many other genera, the cell-body contains, in addition to one or more nuclei, extranuclear granules of chromatin, termed *chromidia,* which may be scattered in the cytoplasm throughout the cell, or may be aggregated in certain regions of the body to form 'chromidial masses' or 'chromidial nets.'"

Regulatory movements between a fragment and a cell were not maintained when a fragment was taken from an individual of one species and placed by the side of an individual of another species. This is in accord with the observations of Wilson (1907), who found that in dealing with dissociated sponge cells, "in contact two masses of the same specific protoplasm tend to fuse," while "unlike specific substances (protoplasms of quite different species) do not tend to fuse." In *Difflugia* protoplasms from two different species do not fuse or coalesce. Also the following quotation from Lillie (1920) can be applied to our findings on *Difflugia:* "If we put two different species of yeast or bacteria into the same culture-medium, each builds up protoplasm of its own kind; *i.e.,* each effects a special predetermined kind of chemical transformation in the materials which it incorporates from the surroundings. Each has the same external materials as its source of supply, but each transforms them in its own specific way, and hence builds up a special kind of protoplasmic structure which, having a special physio-chemical constitution or organization, exhibits corresponding special activities. The term specificity denotes this peculiarity."

This *peculiarity* has been carried a step further in *Difflugia,* for we find that in distantly related individuals of the same species the protoplasm has become so modified in an individualistic way that fusion will not take place between them. Loeb (1920) has recorded observations on the power of tissues to exhibit different reactions to different degrees of family relationship. Our experiments suggest a similar ability on the part of *Difflugia.*

SUMMARY.

1. In *Difflugia* separated pseudopodial fragments are recovered by their cell-bodies, as evidenced by over one hundred experiments. The species included in these observations were: *D. acuminata, D. corona, D. pyriformis, D. spiralis,* and *D. vulgaris.* The distances by which the fragments and their cell-bodies were separated ranged from a few micra to 1,500 micra. The unfavorable conditions under which reunion does not occur are: (*a*) Injury to cell with consequent failure on its part to extrude pseudopods; (*b*) separation from fragment by too great a distance; (*c*) evaporation of water before completion of reactions.

2. The severed fragments are not recovered as food, but enter again immediately into the protoplasmic structure of the cell-body. Such fragments are not reappropriated after they show visible signs of death.

3. In our observations fusion never occurred at the ends of pseudopods, but always took place along an extended mid-region. This indicates a physio-chemical difference between the ends and the middle of such pseudopods.

4. In some cases the severed fragments, which were only ectoplasmic in composition, were observed to take an active part in restitution. Not only did they move toward, but apparently shifted, their line of approach to correspond with the changing positions of their cell-bodies.

5. Fusion has not been observed to take place between two enucleated fragments, even though they be placed in contact.

6. Fusion between an individual of one species and a fragment from an individual of a different species has not been observed. The phenomenon seems to be specific.

7. Observations have been made in which individuals of the same species, obtained from the same wild culture, showed a decidedly negative response toward each other's fragments. Yet in other instances such cross-fusions did occur. This latter suggests the possibility of close genetic origin.

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EXPLANATION OF PLATES.

Plate I.

Fig. 1. *Difflugia pyriformis.* While at position *A* a fragment was cut off and left with spherical contour at position w. As cell-body moved to positions *B, C, D, E, F,* and *G,* fragment *w* changed shape and travelled as an ameboid body along path indicated by *x, y,* and *z*. *z* fused with the side of the upper pseudopod of cell-body at *G*.

Fig. 2. *Diffluga spiralis.* Fragment *c* cut off at 11:38 A.M. Fragments *a* and *b* cut from two pseudopods synchronously at 11:30 A.M. These fragments *a, b, c* now lay with reference to each other and to the cell-body as indicated. In a little time a slender pseudopod of cell-body came down by side of shell and *a* was appropriated. The cell-body was now dragged six diameters of its shell to left from *b* and *c*. By 11:45 A.M. it had taken up *c*. It was then disturbed with a glass rod; but at 11:45¾ A.M. it had emerged and taken up fragment *b*.

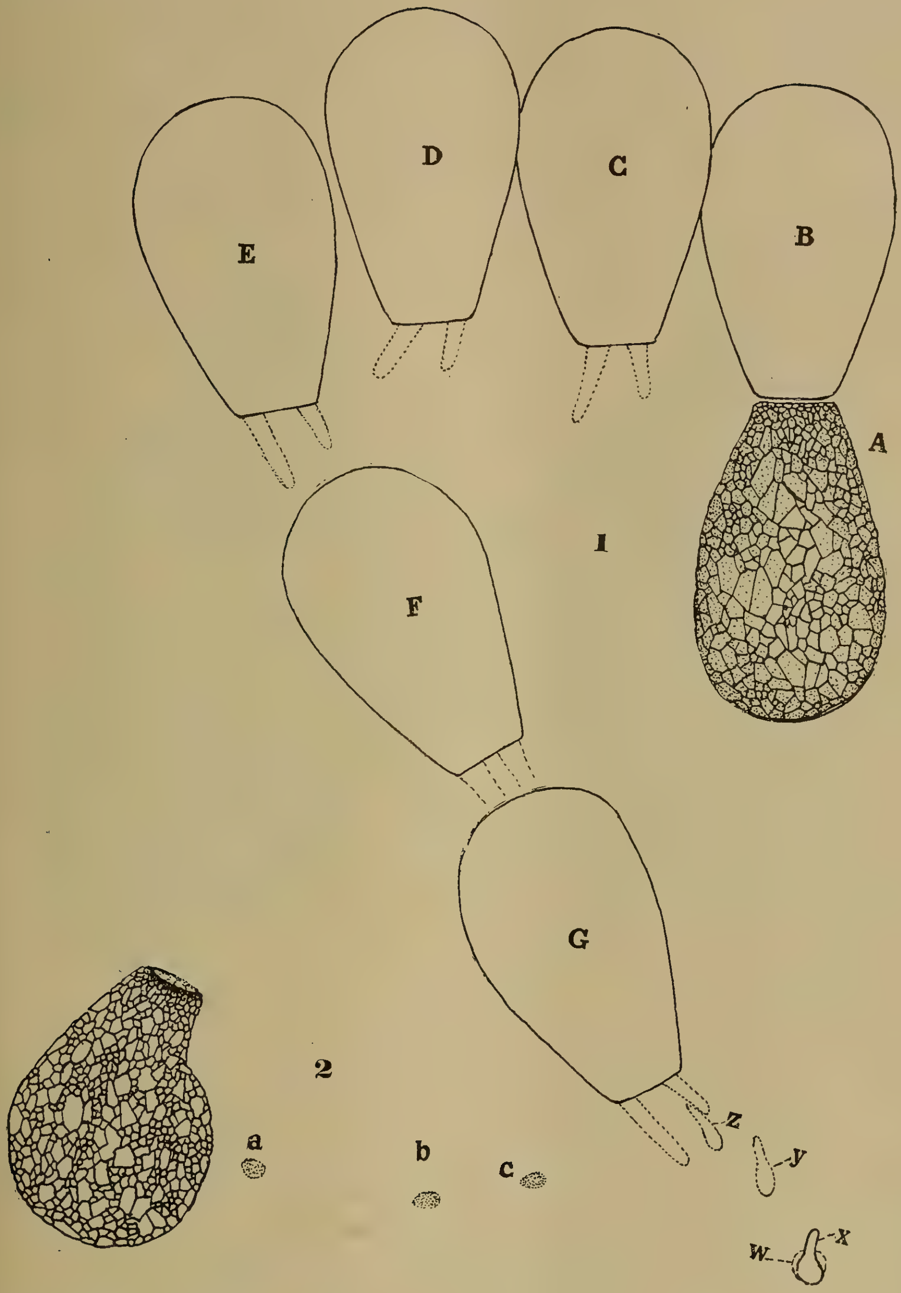
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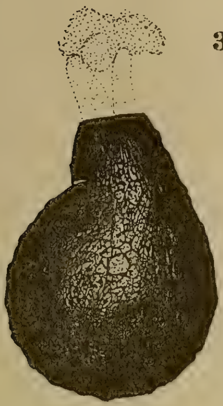
Plate II.

Fig. 3. *Difflugia spiralis.* 3–*A*. Specimen placed in contact with a dead and a nearly dead fragment of its own ectoplasm. The dead fragment was at once rejected, the animal passing it (clinging along upper side of shell at 3-*B*) and dragging the nearly dead (spherical) fragment with it 3-*B*. In time both fragments were rejected.

Fig. 4. *Difflugia pyriformis.* Fragment *a* was whipped off by the sudden retraction of the pseudopod *A*. This fragment lay, of course, in position indicated by end of pseudopod *A*. For graphic reasons it and *b, c, d,* had to be placed below. While fragment *a* lay in this position, it lost its refractive axial rod as in *b;* put out a pseudopod to right as in *c;* assumed contour as in *d*. Next it travelled as an ameboid body to positions *e, f, g*. In the meantime pseudopod *A* had not reappeared and pseudopod *B* was thrown out behind and beyond *g*. When this met *g*, the currents in *g* were reversed and the body of *g* fused with the walls of the cup that *B* had formed about it, as shown at g'; as a result *g* flowed into *B* without leaving any local enlargement of *B*, or being seen as a discrete body within *B*.



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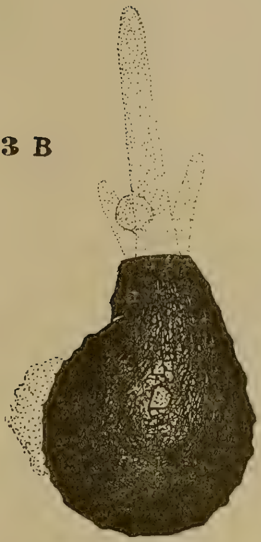

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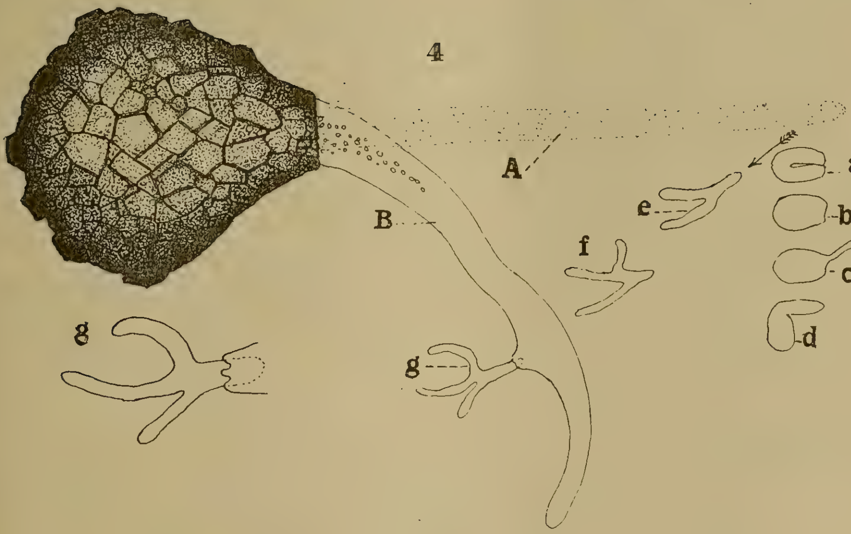
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WM. A. KEPNER AND B. D. REYNOLDS.

Plate III.

Fig. 5. *Difflugia spiralis.* Fragment cut from *a* and dragged to position 1, 1500μ distant, where the fragment had a rod-like contour. Until cell-body had moved through spiral curve along *b, c,* and to *d,* the fragment moved about aimlessly through positions 2, 3, 4, 5, 6, 7, 8, (9 the solid black outline not indicated by a numeral). The fragment and cell-body *d* now travelled towards each other, the former along path 10, 11, 12, 13, 14, and 15, the latter coming to position *e* where the two masses of protoplasm met. × 100.

Fig. 5–A. *a* shows fragment, left at 15 in Fig. 5, now travelling down along pseudopod towards a cup that had been formed; *b* union of fragment and cup just completed; *c* and *d* show the details of the end of this process of restitution. × 250.

Fig. 6. *Difflugia pyriformis.* Fragment *b* had moved from position *b* to *x* towards mouth of shell *A.* Cell-body was now moved to position *B* without disturbing either position or contour of *x.* Immediately after *A* was shifted to *B, x* changed its course and travelled to *y.* Here, unfortunately, the water had evaporated and in adding more water the fragment was lost. × 250.

Fig. 7. *Difflugia spiralis.* Two ectoplasmic fragments taken from an individual and placed side by side as at *m.* Here both are shown as living ameboid bodies. At *n* they are rounding up indicating the approach of death. While at *o* each fragment has become almost spherical and shows no signs of life. × 250.

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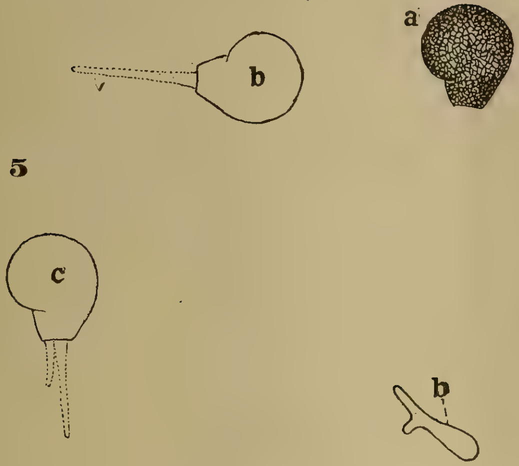

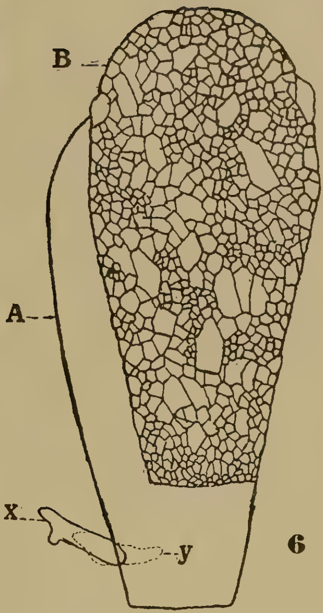

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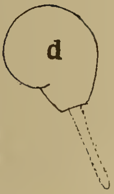

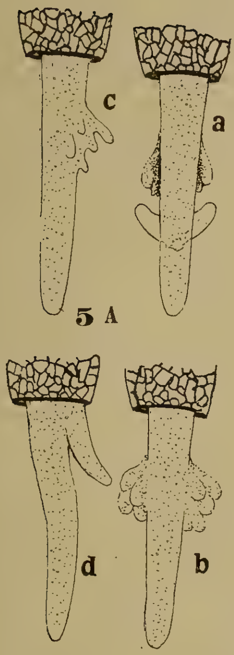

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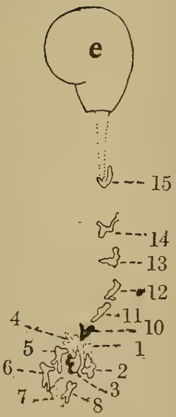
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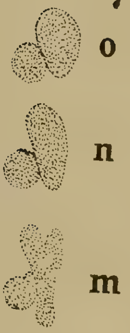
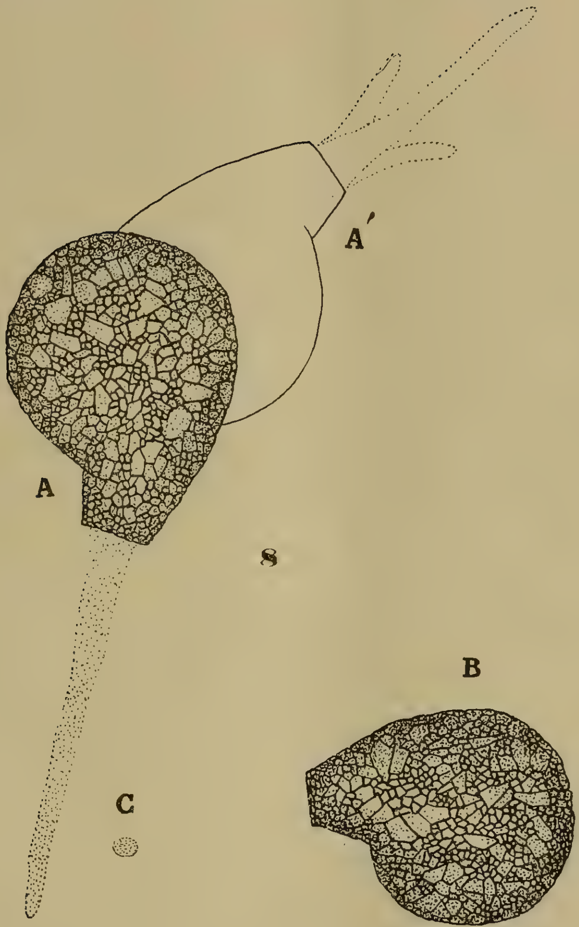


WM. A. KEPNER AND B. D. REYNOLDS

PLATE IV.

FIG. 8. *Difflugia spiralis.* At 11:29 A.M. specimen *A* lay as indicated in figure. Four minutes earlier a fragment had been taken from a pseudopod of this animal *A* and removed from the drop of water. Specimen *B* was brought to position indicated in figure. Fragment *C* was cut from *B* without disturbing *A* even so much as to cause it to retract its pseudopod. *B* was then removed. *A* travelled to position *A′* and moved out of field. It was then brought back to less than half a shell's length from fragment *C*, but though one of its pseudopods passed directly over *C*, it did not react to fragment *C*, nor did this fragment of *B*'s ectoplasm react towards the incomplete cell *A*. × 250.

PLATE IV.



WM. A. KEPNER AND B. D. REYNOLDS

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LILLIE, FRANK R. *Supplementary Notes on Twins in Cattle* . . . 47
GLASER, OTTO. *Copper, Enzymes, and Fertilization* 79

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BIOLOGICAL BULLETIN

SUPPLEMENTARY NOTES ON TWINS IN CATTLE.

FRANK R. LILLIE,

Zoölogical Laboratory, University of Chicago.

Since the publication of my previous papers on the free-martin (1916, 1917) more material has been slowly accumulating. The additional data have confirmed and strengthened my former conclusions, and they also include some observations of a certain amount of intrinsic interest. The present paper, therefore, is a series of supplementary notes on twinning in cattle, with comments on more recent literature.

1. Cases Where the Female of Two-sexed Pairs is Normal.

In my previous paper I concluded that the free-martin is zygotically female, and that its intersexual condition is due to action of the blood of the male twin through anastomosis of the fœtal blood vessels which develops very early; Keller and Tandler (1916) came to the same conclusion as the result of entirely independent studies. This theory receives a crucial test in those relatively rare cases of two-sexed twins in which no such anastomosis develops, for in such cases the reproductive system of the female should be normal. In my previous paper I reported three cases of a normal female twinned with a male out of twenty-four pairs of two-sexed twins. The records of the first two cases (Nos. 8 and 9) were, however, incomplete, and the question of vascular anastomosis was not fully investigated because they were received before any theory on the subject was developed. They were, however, consistent with the above interpretation, for it was recorded in the notes made at the time of collection that the connection between the two chorions was narrow; presumably there was no vascular anastomosis. The third case (No. 40) was, however, a perfect test case in every respect; there was a corpus luteum in each maternal ovary; the two chorions were entirely separate and there was, therefore, no question of vascular anastomosis.

Since this publication two additional cases have been received (Nos. 63 and 64) similar in every respect to case 40. The chorions were entirely separate in these cases also, and in each pair both the male and female possessed normal reproductive organs. In a third case, not previously reported (No. 93), in which the female was also normal, the chorions were connected, but there was no vascular union between the two sides. The notebook records of these cases follow:

No. 63. December 19, 1917; twins in uterus; mother's ovaries missing. Uterus carefully opened; the membranes appear at first sight to be fused in the body of the uterus; but they were really entirely separate, the end of one being merely slightly invaginated over the other, so that they fall apart intact. Length of male and female 22 cm. each; reproductive organs of both entirely normal.

No. 64. February 1, 1918. Twins in uterus; both mother's ovaries present; corpus luteum in each. Uterus carefully opened and chorions found to be entirely separate. In this case the openings from the horns into the body of the uterus were extremely constricted and the chorions were confined to the horns. Male 14 cm. long; female 13.75 cm. long. Reproductive organs of both entirely normal.

No. 93. December 1, 1920. The twins were in the intact uterus; mother's ovaries present; a corpus luteum in each ovary. The chorions when exposed were found to have a very narrow connection, which on examination turned out to be non-vascular. Length of male twin 10.25 cm., of female 9.75 cm. Reproductive organs of both entirely normal.

Keller and Tandler (1916) report six cases, out of a total of 91 cases of two-sexed twins of cattle in which the fœtal membranes were examined, in which the female was normal. In one of these the two chorions were adherent, but not fused in any way; in another there was a narrow non-vascular strand uniting the two chorions apparently similar to my case No. 93. In the remaining four cases the chorions were well united, but the place of fusion was indicated by a narrow strip of white, scar-like tissue completely encircling the chorion; in three of these cases no vascular anastomosis could be demonstrated across the place of fusion; in the fourth case several exceedingly small blood vessels crossed, very different from the typical wide connection between the two circulations. The last case was in the fourth month of pregnancy (size of fœtuses not given). The connections in question may have failed on account of their small size, but it seems more probable that they represented secondary connections formed too late to be effective.

We thus have twelve cases on record in which the female of two-sexed twins of cattle is normal, and in which the conditions of the fœtal membranes are also known—six cases of Keller and Tandler and six cases of my own. In nine of these cases there was certainly no vascular connection; two of my own cases were not satisfactorily investigated with reference to this point, and in one of Keller and Tandler's cases the fine anastomoses that existed were apparently secondary. In all other cases of two-sexed calf twins in which the fœtal blood vessels were examined (29 of the author and 85 of Keller and Tandler) there was anastomosis between the fœtal vessels and the female was definitely intersexual.

The proportion of cases in which the female of two-sexed twins in cattle is normal was found by Keller and Tandler to be 6 out of 91; in my collection it is 6 out of 39; Numan estimates the proportion to be 1 in 8, and Lüer (1913) 6 in 113. (Cited from Keller and Tandler.)

The experiments of Minoura (1921) in which he grafted pieces of testis or of ovary on the allantoic membrane of chick embryos and obtained as a result intersexes of various grades, furnish complete proof that sex hormones can produce precisely the same kind of changes that are found in the free-martin.

2. THE SEX-RATIOS.

The original account involved 54 cases of fœtal twin calves in which the distribution of the sexes was as follows: ♂ ♂ 19, ♂ ♀ 3, ♂ ☿ 21, ♀ ♀ 11; or classifying the normal and intersexual females of two-sexed pairs together, ♂ ♂ 19, ♂ ♀ 24, ♀ ♀ 11. The expected ratios, assuming approximate equality of male and female zygotes in cattle, would be in the proportions of 1 : 2 : 1—*i.e.*, 13.5 : 27 : 13.5. It is obvious that there is an excess of male pairs which is not readily interpretable as due to chance, even though the numbers are relatively small.

I have now 38 additional pairs of fœtal twin calves (Nos. 58–97, excluding No. 66, which was a pair selected after birth, and No. 80, which was apparently monozygotic); the addition of these gives the following distribution of the sexes for all my cases: ♂ ♂ 29, ♂ ♀ 6, ☿ ♂ 33, ♀ ♀ 24; or again combining normal and intersexual females of two-sexed pairs together: ♂ ♂ 29, ♂ ♀ 39, ♀ ♀ 24. Assuming an equality of the sexes in the population dealt with, the expected distribution of sex in the 92 pairs of twins would be ♂ ♂ 23, ♂ ♀ 46, ♀ ♀ 23. It is obvious that there is an excess of male pairs and a deficiency of two-sexed pairs on this assumption. The numbers are not very large, but probably sufficiently so to be significant.

The aberrant ratio for the twins would be satisfactorily explained if the transformation of the free-martin sometimes went so far in the male direction as to render it indistinguishable from a male. I have, therefore, recently studied male pairs with considerable care, but have found no evidences of abnormalities in the reproductive organs; in my experience the structural differences that distinguish a free-martin from a male are always very great. It was natural to expect the greatest modification of the female when both the male and female twin came from the same ovary and developed in the same horn of the uterus, for under these circumstances the vascular anastomosis would presumably be unusually early and extensive. The two cases of this sort which I have, however, show no greater modification of the female than many others (see Sec. 4).

It appeared probable, therefore, that the assumption of approximate numerical equality of the sexes in the population was at fault. I therefore suggested to Mr. J. M. Jewell that he ascertain the sex-ratio in fœtal calves taken from the same slaughter-house. Accordingly the sex-ratio of 1,000 cases taken at random was determined (Jewell, 1921). For our purpose it is sufficient to notice that a sex-ratio of 123.21 was established for the 1,000 cases, including all fœtal ages; the sex-ratio for the sizes included in the twin collection (3.5–30 cm. in length) was found to be 120 (*i.e.,* 120 males for every 100 females; 264 entries).

Taking this figure as the sex-ratio of the population from which the twins came, the expectation of sex distribution in the 92 pairs of twins would be ♂ ♂ 27.37, ♂ ♀ 45.62, ♀ ♀ 19.01. This shows a type of sex distribution similar to that actually found in the twins, which, however, show a sex-ratio of only 111.5 counted as individuals. The approximation of the twin sex-ratio to the expected sex-ratio is, therefore, sufficiently exact when the smallness of numbers is taken into account to exclude the need of any special assumption.

Incidentally it may be noted what a complete demonstration the figures furnish of the underlying assumption that the free-martin is zygotically female, which D. Berry Hart apparently continues to question (1918). In a recent paper Gowen (1921) presents extensive data showing that twins of cattle, whether of the same sex or both, do not resemble one another in color more than full sisters of the same breed. As the latter are, of course, dizygotic, the presumption is that the twins are so likewise, or at least that monozygotic twins in cattle are extremely rare.

3. On Monozygotic Twinning in Cattle.

The embryological criterion for distinguishing dizygotic and monozygotic twinning in cattle would be the occurrence of two corpora lutea in the maternal ovaries in the former case and of one in the latter. Implantation of the fœtuses in one horn of the uterus would also be expected, but this can not be relied upon by itself as evidence, for one ovary often furnishes two ova, both usually remaining in the horn of the uterus on the same side as the ovary. It is also conceivable that two ova derived one from each ovary might lodge in one horn of the uterus owing to passage of one of them from one horn to the other; however, I have found no such case. The existence of a single chorion would also, of course, be postulated in monozygotic twinning, but its value as evidence would be negligible owing to the tendency for originally separate chorions to fuse in cattle.

In the case of cattle, therefore, the embryological evidence for monozygotic twinning is reduced to the occurrence of only one corpus luteum for two embryos of the same sex. One such case (No. 80) occurs in my collection. The chorion is single, resembling in all respects cases in which the usual two corpora lutea are from one ovary; the amniotic cavities were adjacent, but not communicating; the twins were females of about 16 cm. greatest length. The preparation has been preserved complete in formalin to demonstrate these points. This may be a case of monozygotic twinning; but the possibility that the Graafian follicle furnishing the corpus luteum contained two ova, both of which were fertilized and developed, can not be neglected.

My records include 36 cases of twins in which both maternal ovaries were present; of these 35 had two corpora lutea either in both ovaries or in one. In the 90 cases reported by Keller and Tandler there were 88 cases of two corpora lutea and 2 of three corpora lutea. Thus only one case of 126 known cases exhibits one corpus luteum for two embryos. These statistics demonstrate how futile it is to continue to appeal to monozygotic twinning, as D. Berry Hart (1918) does, as the cause of the intersexual condition of the free-martin.

4. The Formation of the Free-martin an "All-or-none" Reaction.

Cases of two corpora lutea in one maternal ovary are apparently not very rare in cattle twins, to judge from the statistics of Keller and Tandler, who found 36 such cases out of 90. I have found only three such cases out of 36 cases where both ovaries were present, but the small number may be due to the fact that the butchers, on whom we relied for selecting uteri containing twins, would be likely to overlook twin pregnancies confined to one horn of the uterus. Cases of this kind in which one fœtus is a male and the other a free-martin are of considerable theoretical interest, because implantation of the twins in one horn of the uterus offers presumably the maximum opportunity for modification of the female.

In all cases in which the twins come from two ovaries it is necessary for the chorionic vesicles to grow entirely through their respective horns of the uterus before they can meet in the corpus uteri, and the fusion that takes place there must occur near the apices of the vesicles, as far removed from the embryos as possible. It is probable that the embryos would be about 15 mm. long before fusion (cf. my earlier paper, p. 389), and we have no data for deciding how quickly vascular anastomosis would become established and effective. When, on the other hand, both ova come from one ovary (Fig. 1) and develop in one horn of the uterus,

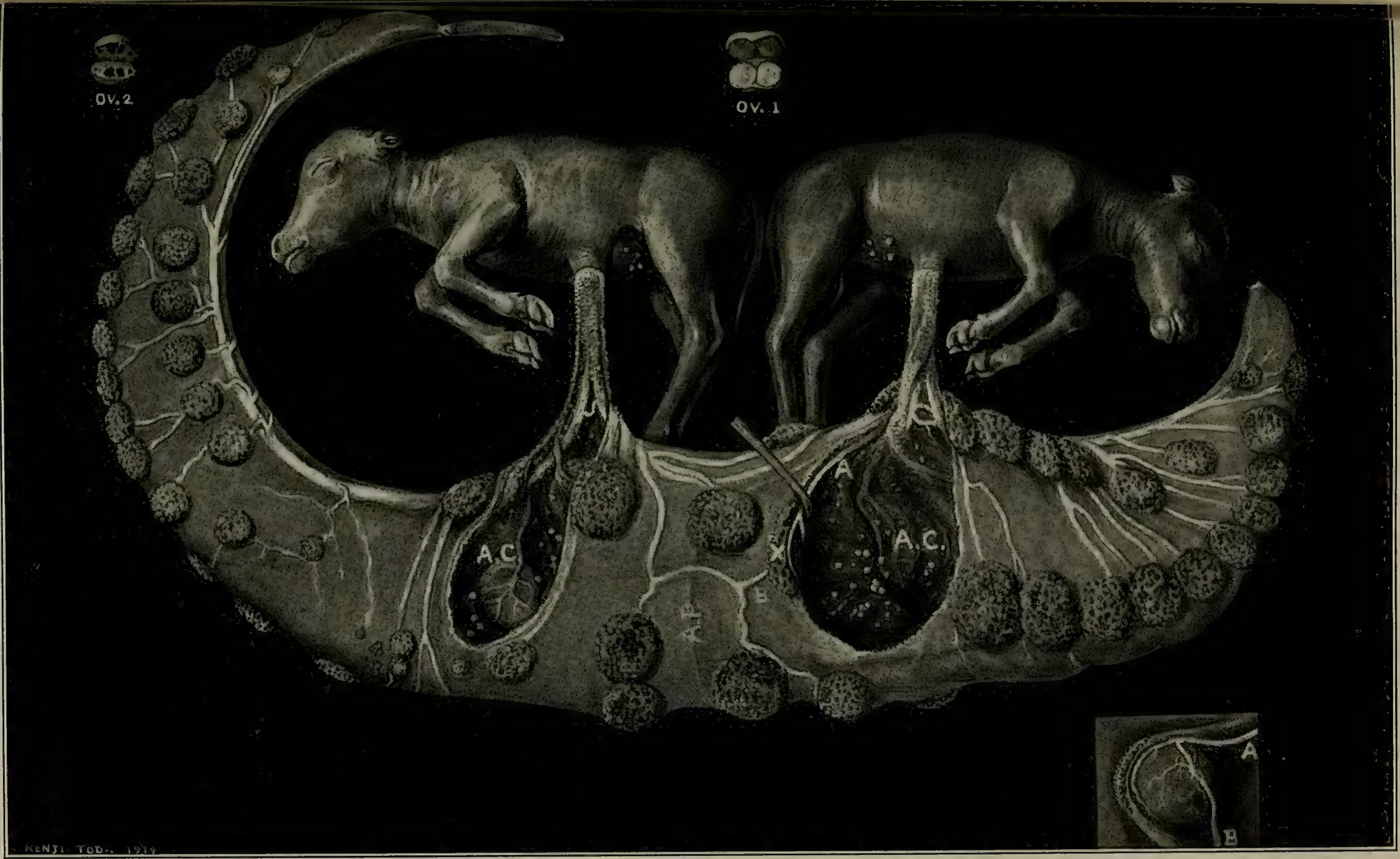

Fig. 1. Case No. 72. Female twins. To illustrate the membranes when both embryos are derived from one ovary. Two corpora lutea occur in ovary 1, none in ovary 2. Both embryos were in one horn of the uterus. Each 28.5 cm. long. *A. P.* shows the position of the partition between the two amniotic sacs. An arterial anastomosis is shown in the insert figure which shows the inner surface of the cotyledon at *X* between the points *A* and *B* of the main figure. (× by 5/22.)

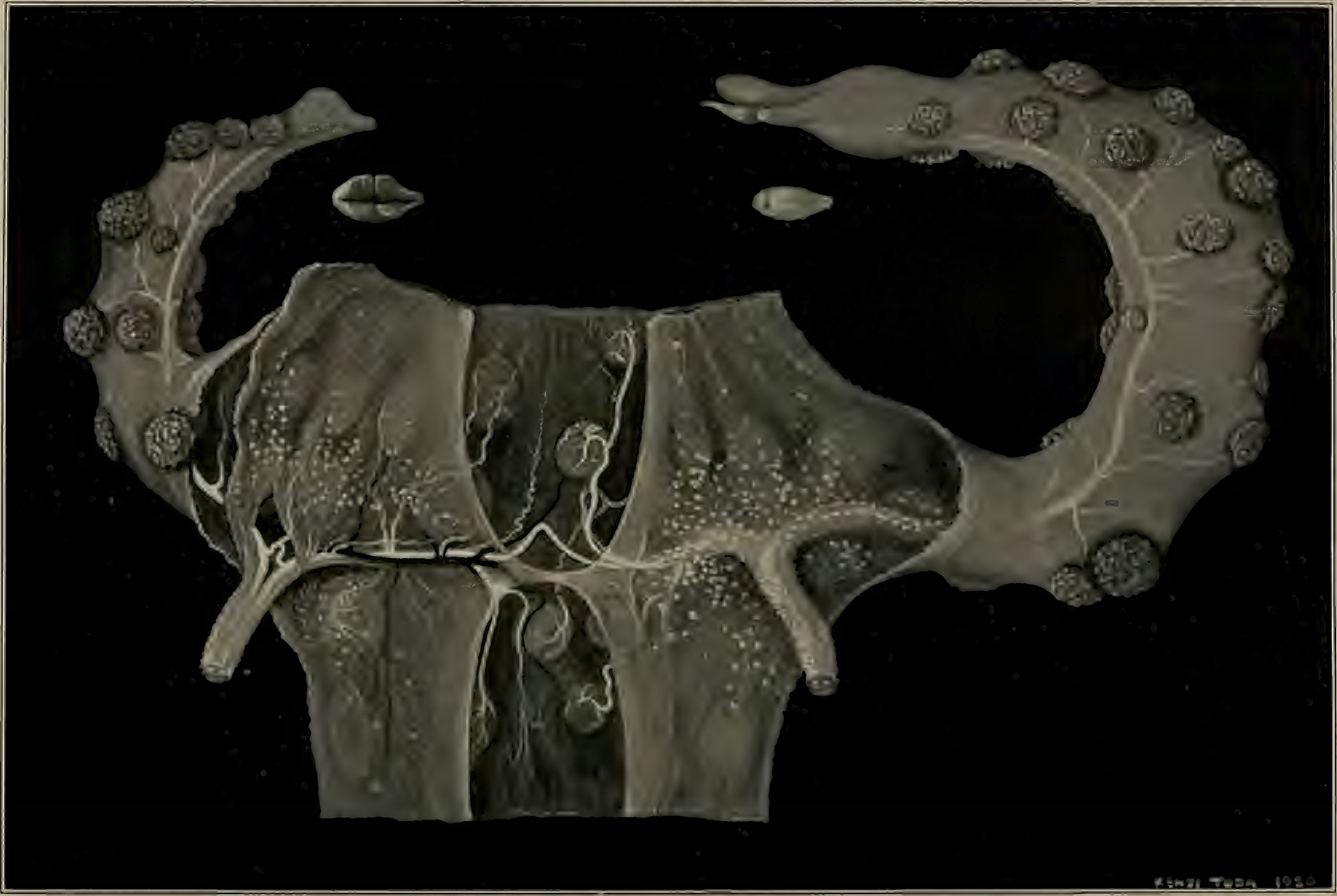

Fig. 2. The membranes of Case 78 described in the text to show the relations of the two amniotic sacs and the strong anastomosis between the middle [illegible]

fusion may take place at a much earlier stage; and vascular anastomosis may date from the time of extra-embryonic circulation. The approximation of the embryos in the common chorion may give some indications of the manner and time of fusion, and the vascular relations will also throw some light on the same questions.

I have two cases of this kind (Nos. 78 and 97) in which the fœtuses are of opposite sex, but in neither case is the modification of the free-martin particularly extreme. They are so alike that the description of one of them (No. 78) will suffice. Fig. 2 is from an oil painting showing the membranes; the very direct arterial connection between the two umbilical cords will be noted. The two amniotic cavities are so close together that the walls are in contact at one place. The fusion of the two original vesicles was presumably, therefore, side by side and very early, for all overlapping parts have disappeared without leaving any trace.

If I were to venture to reconstruct the probable history of this case, I would say that the fusion was probably complete at least by the 10-mm. stage, and vascular anastomosis established at the same time. The reasons for this opinion are the extent to which the membranes of a 10-mm. embryo fill up one horn of the uterus (see Lillie, 1917, p. 389) and the readiness with which fusion of the membranes takes place (specifically in cattle) when they are in contact. If this opinion is correct, the vascular anastomosis in this case dates from long before the period of beginning of sex differentiation.

The anatomy of the reproductive organs of the free-martin is shown in Figs. 3*A* and 3*B*. If these figures be compared with figures of free-martin anatomy given in my 1917 paper and with Figs. 4 and 5, it will be seen that the extent of the modification is less than the average in certain respects. Thus the cornua and corpus uteri are retained in this case, though very rudimentary compared with the normal; in probably the majority of free-martins they are entirely absent (cf. Fig. 5); the Wolffian ducts are also more slender than in most free-martins, and the gubernacula less typically developed (cf. Fig. 4). The gonads are exceptionally minute, which would appear to indicate less than the average stimulation of the male homologues in the original ovary out of which the free-martin gonad is entirely composed (cf. Chapin, 1917; Willier, 1921).

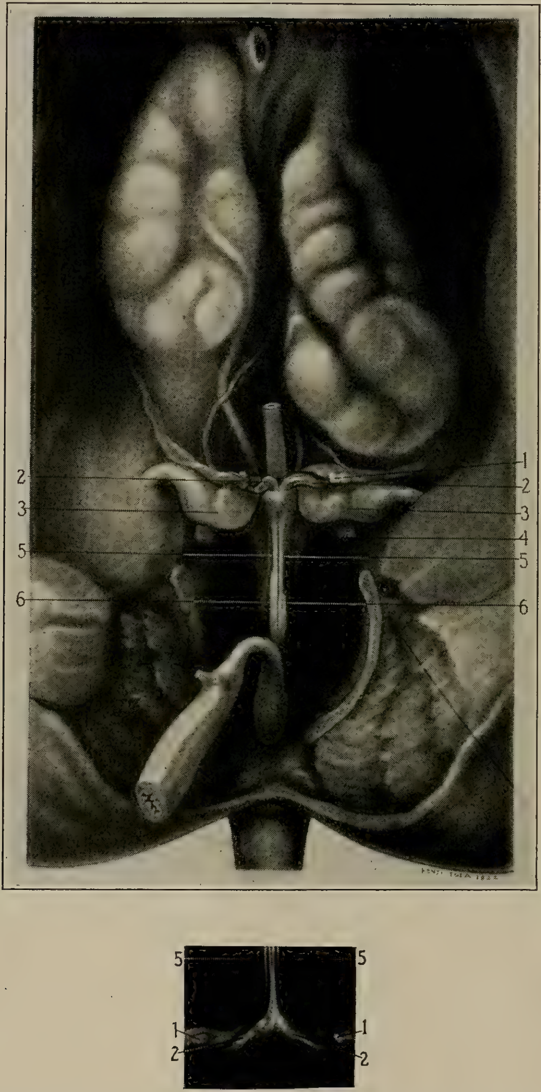

FIG. 3. Anatomy of the urinogenital system of the free-martin of Case 78, 32 cm. long. Described in the text. Greatest length, 32 cm. *A*. The entire system. *B*. The urinogenital cord has been cut across in front of the seminal vesicles and turned over anteriorly so as to expose the dorsal surface. 1. The exceedingly minute gonad; 2. Cornua uteri; 3. Gubernacula invaginated into the body cavity; 4. Cut end of umbilical artery; 5. Wolffian ducts; 6. Very slender seminal vesicles. Natural size.

The idea that I have expressed elsewhere (1917) that the variable degree of intersexuality exhibited by free-martins may be a function of the time of onset, intensity, and perhaps duration of action of the male sex hormones requires qualification or sharper definition. The case we are considering would seem to have afforded the maximum opportunity in these respects, without the maximum result following. It should, however, be borne in mind that no one has yet investigated the stages of the free-martin from

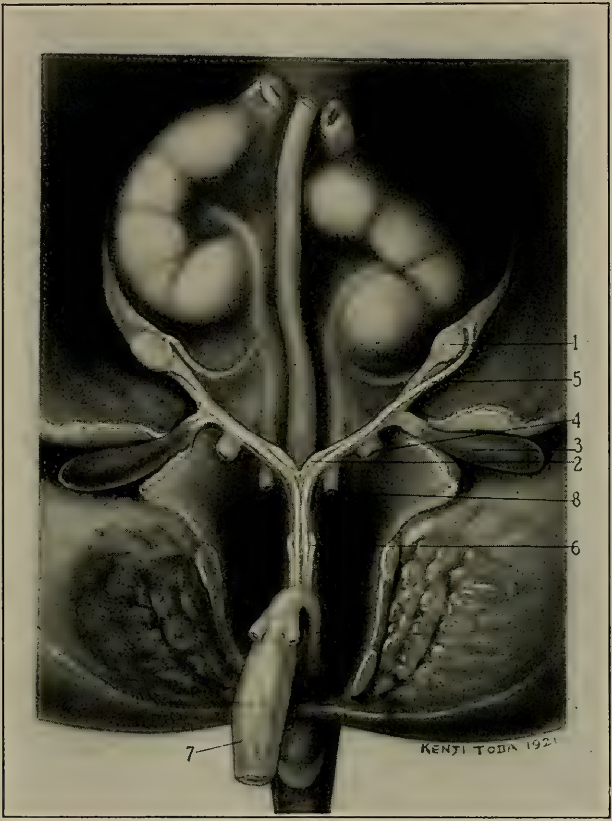

FIG. 4. Anatomy of the urinogenital system of the free-martin of Case 81, 14 cm. long. Described in the text. Greatest length about 14 cm. 1. Gonad; 2. Rudiment of cornua uteri; 3. Gubernaculum; 4. Cut end of umbilical artery; 5. Wolffian duct; 6. Seminal vesicles; 7. Allantoic stalk reflected; 8. Ureter. (× 2.)

32 cm. length to birth (about 100 cm.), and that the fœtal conditions have not, therefore, been adequately correlated with adult conditions. It would seem that the major portion of the growth of the gonad of the free-martin occurs in these missing stages, and

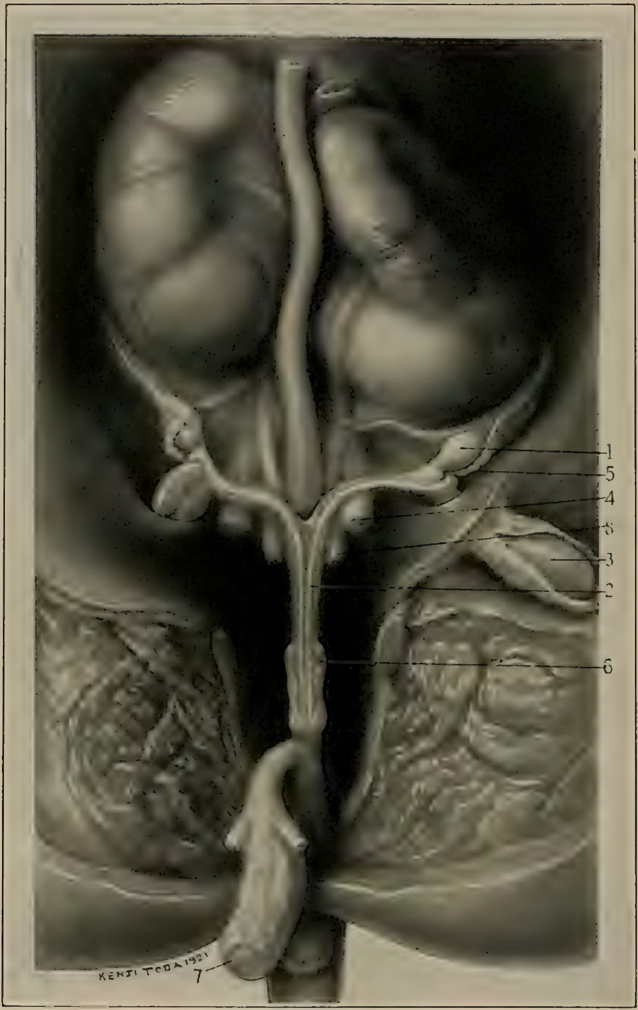

Fig. 5. Anatomy of the urinogenital system of the free-martin of case 94, 19.25 cm. long. Described in text. Greatest length about 19 cm. 1. Gonad; 2. Wolffian duct; 3. Gubernaculum; 4. Cut end of umbilical artery; 5. Wolffian duct; 6. Seminal vesicles; 7. Allantoic stalk reflected; 8. Ureter. (× 2.)

it is possible that in the present case the gonad would have grown considerably. But it could not have passed out of the body cavity, for the gubernacula did not enter the body wall in this case. And it is impossible that a male type of external organs could have developed in this case, for the definitive female type was already formed.

Cases at the other extreme from the present one also throw some doubt on the merely quantitative explanation of the degree of intersexuality. I refer to cases where the vascular interconnection is very delicate. One case of this kind, not previously reported, is especially striking (No. 81). In this case I at first doubted the existence of an actual arterial anastomosis, for the injection was not very successful. A careful examination, however, showed an exceedingly delicate artery from the male side passing far into the territory of the female chorion and there apparently anastomosing with an artery from the female. The reproductive system of the free-martin in this case (stage of 14 cm.) was typically intersexual (Fig. 4) and similar in all essential respects to the case shown in Fig. 15 of my former paper: gonads reduced, ducts of the male type with rudiments of the cornua uteri persisting, however, gubernacula present, etc. Indeed, this case is certainly more modified than No. 78.

Keller and Tandler (1916) describe a case in which the vascular arrangements found seemed inadequate to account for the typically deformed condition of the reproductive organs of the free-martin. In this case they state that no strong vascular anastomoses were demonstrable, nevertheless the female exhibited the typical malformation of the reproductive system. They add that a welt-like connection similar to that which usually carries the connecting vessels was found between the main vascular trunks on the two sides, but that it possessed no lumen. They consider this as pointing toward the possibility of an earlier typical connection that became interrupted secondarily.

All free-martins that have been described depart very widely from the female anatomical plan, and also very widely from that of the male. If we were to imagine a sex scale of, say, twenty points, in which points 1, 2, and 3 on the left include the normal female range and 18, 19, and 20 on the right the normal male range, then I would say, as a matter of quite arbitrary judgment, that the free-martins would occupy points 8 to 13, inclusive, possibly encroaching in some doubtful cases on points 14 and 15. The free-martins, in other words, form an exceedingly well-defined group without intergrades to normal female or normal male. Within the group of free-martins there is considerable range of variation which may be classified according to degree of approximation toward the male type, as B. H. Willier (1920) has shown for the gonads. The ducts also would permit of a similar classification, though it is not certain that the classification by gonads and ducts would always agree.

The absence of intergrades to the female side, and the absence of correlation between the size of the vascular anastomosis and the degree of intersexuality, indicates an "all-or-none" type of reaction in formation of the free-martin; presumably, therefore, the differentiating action concerns a rather definitely delimited period of development and does not require a maximum amount of the male hormones. If this be so, variations of the quantity of the hormones within wide limits would not be significant for the typical reaction. But variation in time of onset with reference to the preceding degree of differentiation of the ovary and other organs of the female may be sufficient to explain in part the range within the free-martin series (cf. Willier, 1920). The matter is discussed further in section 6 (p. 70).

It is interesting to note that Pézard (1920) concludes that the morphogenetic effects of transplanted testicular fragments on castrated cocks also follows an "all-or-none" law; "as soon as the functional threshold is passed, whatever may be the mass of the active gland, a cock takes on as a whole its secondary sexual characteristics. It appears that these manifestations can not be fractionated."

5. On the Occurrence of Seminal Vesicles in the Fœtal Free-martin.

In my study of the anatomy of the fœtal free-martins (1917) the existence of seminal vesicles in the stages in question was doubted, although they appear in the males in much earlier stages. On the other hand, seminal vesicles appear usually to be present after birth in free-martins, and I therefore assumed that their appearance in the free-martin was probably belated. A reëxamination of the material, however, shows that they are always present in fœtal free-martins in which the lower part of the Wolffian ducts is developed. Fig. 5 from a specimen hitherto not recorded (case No. 94) shows the typical condition in a free-martin with well-developed Wolffian ducts.

Reëxamination of the specimens figured in my 1917 paper shows similar structures, sometimes more slender, in those represented by Figs. 14, 15, 17, 18, 20, 21, 22, 24, 25, and 27. On the other hand, the specimen shown in Fig. 26 had no Wolffian ducts and no seminal vesicles. The other specimens figured were not available for reëxamination. The specimens were drawn correctly, as dissected at the time the drawings were made; it required further dissection to reveal the seminal vesicles (cf. Fig. 5 of this paper with the figures of the 1917 paper).

This correction removes the necessity for the special assumption made in the 1917 paper that these organs develop out of their due time.

6. The Earliest Stage of the Free-martin.

The smallest free-martin hitherto described is 7.5 cm. greatest length, case 19 of my 1917 paper. The gross anatomy was described and figured by myself, and the histology by Miss Chapin (1917). The normal female characters are highly modified at this stage; the ovary exhibits a serous covering and albuginea similar to that of the male, absence of the ovarian cortex, and presence of the medullary components of the ovary. In the normal ovary of the same stage the cortex is highly developed, though Miss Chapin states that the cords of Pflüger are not yet separated from the germinal epithelium. The Müllerian ducts of the free-martin have begun to degenerate similar to a normal male of corresponding age (Chapin, p. 459). It is, therefore, apparent that the modifications of the free-martin must start at a considerably earlier stage.

From a study of the chorionic vesicles of normal single pregnancies I concluded that the opportunity for fusion of embryonic membranes from a two-sided twin pregnancy is present from the

10-mm. stage of the embryos, and for vascular anastomosis from the 19-mm. stage, or slightly earlier, because the allantois passes completely through the body of the uterus at that time. This antedates the beginning of visible sex differentiation which I estimated then at about 25 mm., confirmed since by a much more extensive study.

These considerations indicated the possibility of exchange of blood between twin embryos before the beginning of sex differentiation, but it was not demonstrated that this actually occurred, nor yet how early the blood of the male had any sex hormone contained in it.

The case about to be described (No. 62) gives a fairly definitive answer to these problems. The point of chief interest, demonstrated below, is that the hormones in the male blood have produced a practical inversion of the ovary of the female twin at a stage of 3.75 cm., and must hence already have been active for some time. The weight of a male embryo of 3.8 cm. is 3.65 gr.; the weight of its two gonads is 0.0025 gr., or about one fifteen-hundredth that of the embryo. The quantity of interstitial tissue can be only a small fraction of the total mass of the gonads. Nevertheless the secretion of this relatively minute fraction of tissue diluted by the total blood of both embryos is sufficient during growth of about one centimeter in length to reverse the normal course of development of the ovary of the female. I know of nothing that gives a more profound impression of the chemical aspect of development than this demonstrable fact.

Case No. 62: ♂ 4 cm. greatest length, ☿ 3.75 cm.; one in each horn of the uterus; collected in February, 1917. Only one of the maternal ovaries was present and it contained a single corpus luteum. The two chorions were fused so completely that the actual place of fusion can be determined only approximately. Injections of the umbilical arteries with a starch mass did not pass from one side to the other, but at least one perfectly distinct, though very fine, arterial anastomosis by side branches of the main arteries of the two sides could be demonstrated under the binocular.

The two embryos were at first diagnosed doubtfully as males and hence received no further attention for a long time, until I had become familiar with definite external characteristics that enabled me to distinguish the sexes with certainty as early as 3 cm. greatest length. They were then readily diagnosed as male and female, respectively, in their external characters. They were carefully studied and compared with normals, with the result that the reproductive system of the female was found to be already definitely modified in the characteristics about to be described.

Fig. 6 shows the external organs of the free-martin. The short length of the perineum and the development of the urinogenital aperture are distinctive female characteristics. In the male the perineum is several times longer and marked by a ridge; there is no urinogenital aperture in the male, and the phallus is placed more anteriorly with reference to the scrotal sacs, which are entirely similar in the two sexes.

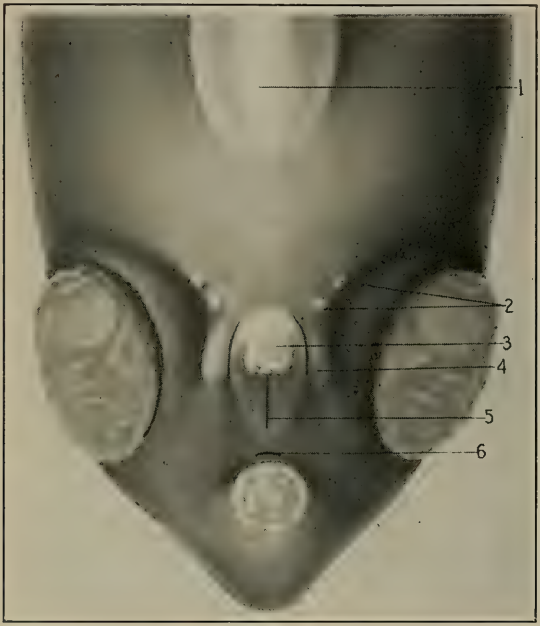

Fig. 6. Free-martin 3.75 cm. greatest length; Case No. 62. × 6. 1. Umbilical cord; 2. Teats; 3. Phallus. 4. Homologue of scrotal sac of male, formed in all females; 5. Urinogenital aperture; 6. Anus.

Figs. 7, 8, and 9 show the internal urinogenital organs of the free-martin (Fig. 7), of its male twin (Fig. 8), and of a normal female 3.6 cm. greatest length (Fig. 9). These figures are drawn with the utmost care as to sizes and proportions of the parts. The much smaller size of the gonads of the free-martin is apparent at a glance. The actual measurements giving the average of the two sides in each case are: free-martin — 1.727 mm. × 0.818 mm.; male twin — 2.622 mm. × 1.346 mm.; normal female — 2.568 mm. × 1.346 mm. The third dimension exhibits at least an equal difference, as may be seen by comparison of the outlines of cross-sections, Fig. 10, *a*, *b*, *c*, drawn at identical magnifications. The male and female normal controls may be regarded as typical for size of the gonads at this stage, for I have examined several other

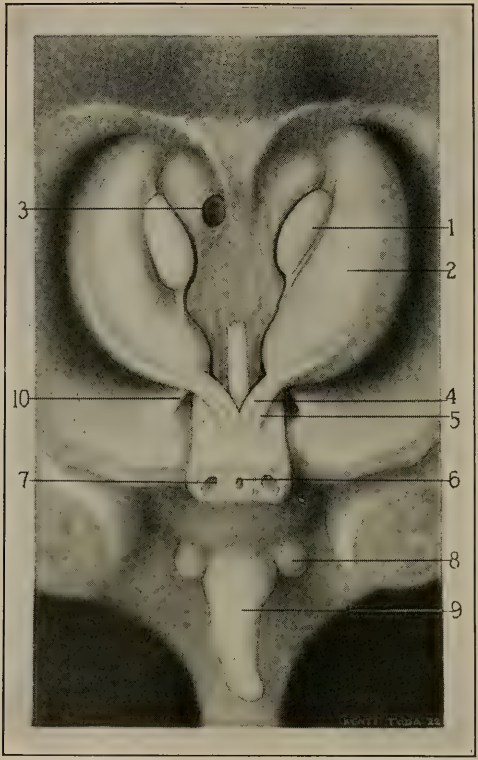

FIG. 7. Internal urinogenital organs of free-martin No. 62. 3.75 cm. greatest length, × 6. 1. Gonad; 2. Wolffian body; 3. Vena cava inferior; 4. Müllerian duct; 5. Wolffian duct; 6. Allantois; 7. Umbilical artery; 8. Homologue of scrotal sac of male; 9. Phallus; 10. Inguinal ligament ("round ligament" of uterus).

specimens of each sex between 3 and 4 cm. in length for comparison. The hormone of the male has thus already operated so as to inhibit the growth of the gonad of the female to a very marked extent.

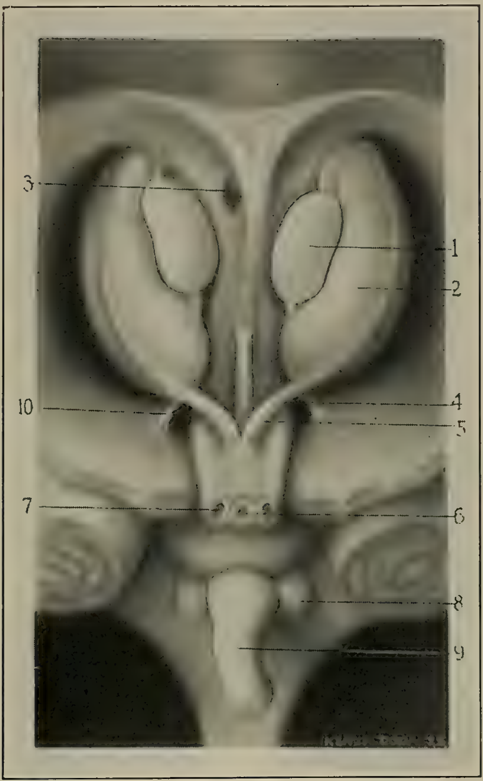

FIG. 8. Internal urinogenital organs of male twin No. 62. 4 cm. greatest length, × 6. 1. Gonad; 2. Wolffian body; 3. Vena cava inferior; 4. Müllerian duct; 5. Wolffian duct; 6. Allantois; 7. Umbilical artery; 8. Scrotal sac; 9. Phallus; 10. Inguinal ligament (part of gubernaculum).

The inhibition in growth is accompanied also by inhibition of the characteristic differentiation of the ovary, though apparently not as yet, to any marked degree, by stimulation of the male homologues in the ovary. In Figs. 11, 12, and 13 comparison is made between the histological structure of segments of those parts of the section outlines of Fig. 10 indicated by the reference numbers. In the male (Fig. 12) the germinal epithelium is already reduced to a single layer of nuclei, the albuginea has undergone

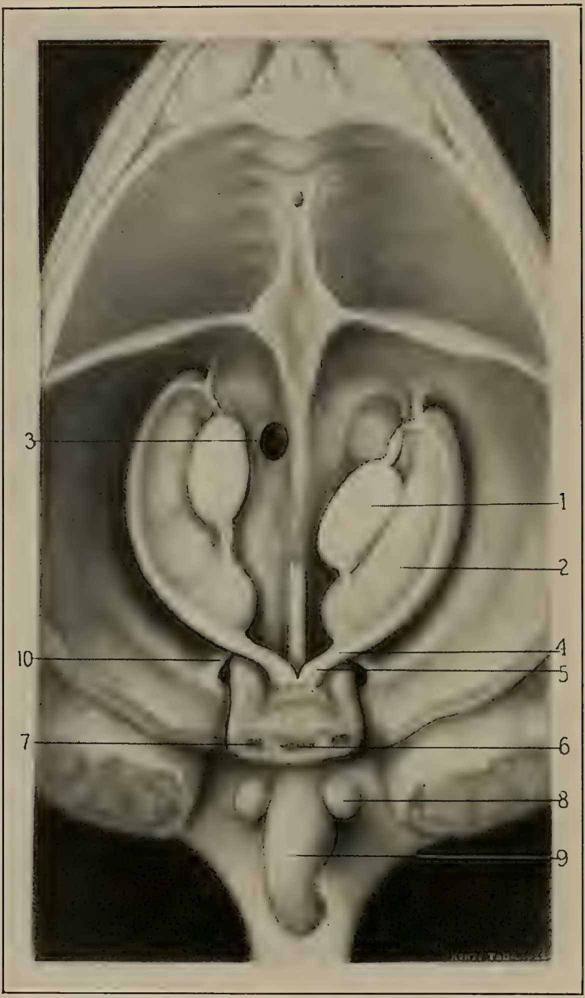

FIG. 9. Normal female, 3.6 mm. greatest length (No. 54*c*) × 6. 1. Gonad; 2. Wolffian body; 3. Vena cava inferior; 4. Müllerian duct; 5. Wolffian duct; 6. Allantois; 7. Umbilical artery; 8. Homologue of scrotal sac of male; 9. Phallus; 10. Inguinal ligament.

definite fibrous differentiation, and the primary sex cords are forming definite tubule rudiments with abundant interstitial tissue between. In the female (Fig. 13) the germinal epithelium (about 140 μ in thickness) is in very active proliferation forming several, to many, layers of nuclei. Cords of Pflüger are definitely indicated in the new cortex thus established. The primary albuginea contrasts with that of the male by its more embryonic type. The primary sex cords are not sharply differentiated from the stroma of the medulla. The free-martin (Fig. 11) shows the same three layers. The primary albuginea and the medulla do not differ much from those of the female, but the preservation in formalin was not good enough for detailed comparisons on this point. The germinal epithelium is thicker than in the male, but only about one fifth on the average of the thickness of the female. It is, indeed, not as thick as that of a normal female of 3.0 cm. and contrasts

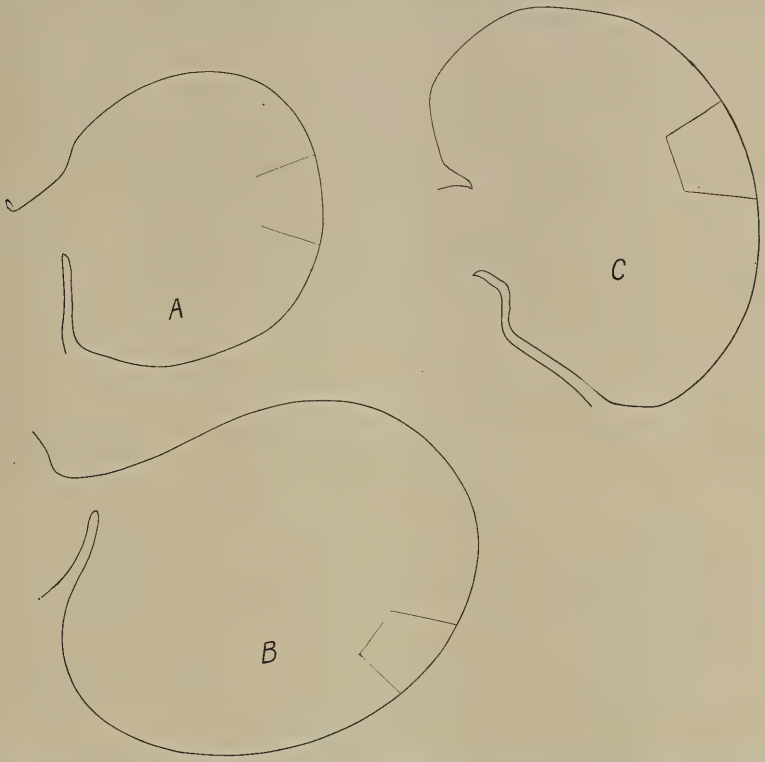

Fig. 10. Outlines of cross sections through center of the gonad, (*a*) of free-martin No. 62, (*b*) of male twin No. 62, (*c*) of normal ♀ No. 54*C* illustrated in Fig. 9.

also with it in the much sparser distribution of the nuclei, evidence of complete cessation of its activity.

It would thus appear that the characteristic proliferation of the ovary was halted in the free-martin at a stage not later than 3.0 cm. As will appear from the work of my student, K. F. Bascom, soon to be published, recognizable differences between the testis and ovary, concerning the germinal epithelium and albuginea, may

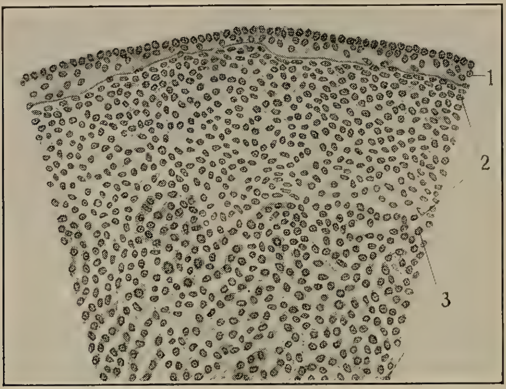

Fig. 11. Detail of area designated in Fig. 10*a*. Free-martin, No. 62. 1. Germinal epithelium; 2. Albuginea; 3. Primary sex-cords (medulla).

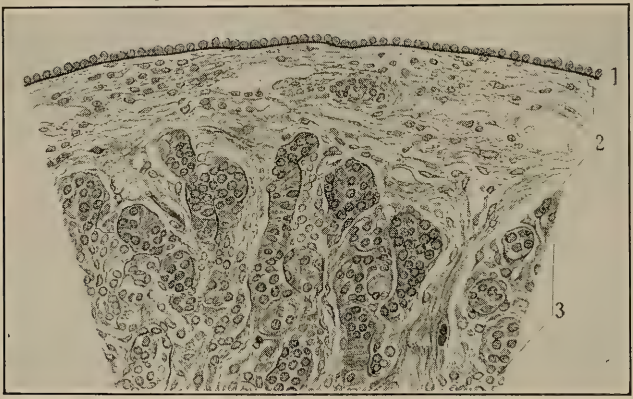

Fig. 12. Detail of area designated in Fig. 10*b*. Male twin, No. 62. 1. Germinal epithelium; 2. Albuginea; 3. Primary sex cords (seminiferous tubules) and interstitial tissues.

be traced back to the stage of 25 mm. at least. Comparison of these leads to the conviction that in this free-martin ovarian differentiation had begun, and that modification did not date from a stage in which the prospective differentiation of the gonad, whether as ovary or testis, is still indistinguishable. However, the work of Chapin (1917) and of Willier (1920) has shown that in later fœtal and post-natal stages nothing remains comparable to the cortex of the ovary.

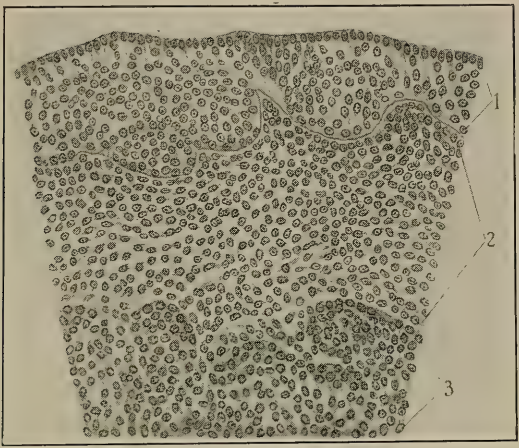

FIG. 13. Detail of area designated in Fig. 10*c*. Normal female No. 54*c*. 1. Germinal epithelium (cords of Pflüger); 2. Albuginea (primary); 3. Primary sex-cords (medulla).

The ostium tubæ abdominale of the normal female is conspicuous at the anterior end of the mesonephros (Fig. 9). It lies in the same position in the normal male (Fig. 8), though it is less conspicuous. In the free-martin (Fig. 7), on the other hand, it can not be seen in the dissection, and was found in sections a considerable distance from the anterior end of the mesonephros, as though the Müllerian duct had failed to grow in length after its formation, and thus its anterior end had become displaced. For the rest there is practically no difference in the ducts in the two sexes; and naturally none was to be expected, nor was any found, in the free-martin.

The feature of most general interest in this case is the demonstration that a specific sex hormone is being produced by the male, presumable from its testis, at a period very closely approximated to the beginning of visible sex differentiation, which has a specific inhibitory action on proliferation of the germinal epithelium and on the rate of growth of the ovary as a whole.

This case is an isolated one so far as the early stage is concerned. There are, therefore, certain restrictions in generalizing from it. The other evidence that we have demonstrates, however, that the general relations in this case are typical. But it is probable that actual vascular connection between the two embryos may, in some cases, be established earlier, and, in other cases, somewhat later. We considered in section 4 the idea that the quantitative aspect of the vascular interchange is probably not an important factor in the degree of intersexuality of the free-martin; that we are dealing, in other words, with a reaction of the "all-or-none" type. So far as we can see, then, the principal factor, so far as hormones are concerned, in determining the range of variation within the free-martin series is probably the time, in relation to the early stages of sex differentiation, at which vascular interchange is established. But to supplement this, I believe it is necessary to assume that different individuals vary in the state of balance of the zygotic sex factors, and this may influence the quantitative effect of the hormone factor.

It is probable from these results that the ovary does not produce a sex hormone at such early stages. If it were doing so, some reciprocal effect upon the male ought to be observed in some cases at least. The histological evidence is just as negative as the physiological in the case of the ovary, while in the case of the testis fully differentiated interstitial cells are found from the 3 cm. stage of the embryo on (Lillie and Bascom, '22). Mr. Bascom will deal with this subject in detail in his study, but I may here call attention to the fact that all students of ovarian hormones locate the cells of origin in the cortex of the ovary, and that this part of the ovary is in its earliest state of origin in the stages in question, and exhibits as yet no differentiation of cells of internal secretion.

Moore (1921) and Sand (1919) have denied Steinach's idea of a mutual antagonism of testis and ovary of mammals by showing that the coexistence of both gonads in the same body is possible. The present results show, however, that prior to the formation of the ovarian hormone the testicular hormone is antagonistic to growth of the ovary. Presumably, therefore, the normal growth of an ovary in the presence of a testis, as in the experiments of Moore and Sand, depends upon a protective action that the elaborated ovarian hormone exerts against the testicular hormone. I do not think that Moore (1921, p. 168) is entirely correct in his opinion that hormone action is never characterized by inhibition, and that in the case of the free-martin the transformation of the gonad is due solely to the stimulating action of the male hormone. The gonad of the free-martin is unquestionably inhibited not only in its total growth, but also in its histogenesis by the male hormone; later yet the growth of the Müllerian duct is halted and degeneration also sets in owing to the presence of the male hormone. It is not until a still later period that the stimulating effects of the male hormone on the sex cords and rete of the gonad and on the epididymis and Wolffian duct of the free-martin become apparent. There is certainly an element of truth in the contention of Steinach, Lipschütz, and others that the sex hormones inhibit heterologous sex characters in addition to stimulating the homologous ones.

The great and important advances made in recent years in the study of sex hormones have led certain authors to extreme positions concerning their economy in the organism. Lipschütz (1919, p. 390 ff.) has given the boldest statement in his theory that the embryonic soma is primarily asexual, and that sex characters are secondarily imposed upon it by the differentiation of a male or female "puberty gland." In the discussion of this statement we should no doubt run foul of definitions. Whether it is to be understood under the presupposition of the zygotic *determination* of sex or not is not clear. If so, it confines itself to the phenomena of sex *differentiation*. The primary differentiation, therefore, which itself requires explanation, would be presumably that of the misnamed "puberty gland." This is, however, demonstrably not the case (Lillie and Bascom, '22). Moreover, if there were no other factor at work in determining the sex differentiation of embryonic primordia than the specific sex hormone, it is difficult to understand why the free-martin, which receives only male sex hormones, should not become completely male. It is obvious that the male hormone is acting against resistance in the female soma; moreover, this resistance is not that of already differentiated parts, for the hormone is introduced before sexual differentiation; it is rather a constitutional resistance native to the determined sex. The phenomena can be understood only on the assumption that the zygotic sex-determining factors are also sex-differentiating factors in mammals as in insects. These factors are reinforced very early by hormone production in the male of mammals, but relatively very late in the female. Such reinforcement can be thought of in the sense of an autocatalytic reaction (as suggested by certain writers, cf. Goldschmidt, R., Huxley, J., 1920), in which the hormones are regarded as by-products of the sex-determining factors present in the zygote, that accelerate the progress of the differentiation.

General Discussion.

On account of certain misunderstandings that appear in the literature since publication of my 1917 paper, I would like to point out the limitations of the theory of the free-martin. I have so far confined the explanation to the bovine free-martin, though indicating the possibility that it may have a wider application. Minoura's experiments (1921) confirm the general applicability of the principle. The explanation is not supposed, however, to apply to all cases of hermaphroditism in cattle; it is not only possible, but probable, that certain cases rest upon a zygotic basis, for hermaphroditism is reported for single births in some cases. The possibility, therefore, exists of an accidental combination of such a case with twinning, though it would necessarily be very rare. There is, however, a sufficient basis for receiving with a certain amount of skepticism cases alleged to be due to twinning in which the history is based on statements of farmers, and not on the personal observations of the investigator. Most of the cases in the literature are, as a matter of fact, of this nature. There is no good reason for rejecting them so long as they are conformable to cases known to be due to twinning; but when they are quite unconformable, especially if they involve extensive modifications of the external parts, judgment may be reserved until proof is forthcoming that such modifications may be due to twinning.

I regard the explanation of the bovine free-martin which I have given as proved by the large body of evidence accumulated by Keller and Tandler and by myself. Esther Rickards and F. Wood Jones (1918) find unconformable cases in goats described by themselves and others and conclude that these "show the unsoundness of Lillie's theories": A pair of apparently female goat kids turn out to be quite identically predominantly male in their anatomy; "it is affirmed of a celebrated he-goat 'that one season *every* kid he sired, eleven in number, was a hermaphrodite'" (quoted from Davies, 1913). Such cases imply a genetic foundation which certainly deserves study and promises interesting results, but they obviously do not have any bearing at all on the bovine free-martin.

Keller and Tandler (1916) investigated the membranes in four cases of triplets and two cases of twins in goats. In two of the triplet cases three corpora lutea were found in each case, and in one of the twin cases there were two corpora lutea. The ovaries were not available in the other two cases. The chorions were fused, but the individual fœtal circulations were separate, as in sheep (cf. Lillie, 1917). The fœtuses of four of these cases, which included both sexes, were examined and found normal in the anatomy of their reproductive organs. These findings thus confirm, as far as they go, the idea that hermaphrodites in goats are not usually a result of twinning as such; but they do not exclude the possibility of the occasional occurrence of intersexual conditions due to hormones. It is obvious that goats are not favorable material for the investigation of the action of sex hormones in fœtal life.

Hartman (1920) suggests that certain cases of hermaphroditism in mammals may be interpreted as "reciprocals" of the free-martin—that is, cases in which the male of a two-sexed pair is modified in its sexual development by hormones of the female. The evidence that he offers is of the slenderest description. Moreover, if, as seems probable, the female in mammals does not produce sex hormones during the early part of fœtal life, the possibility of "reciprocal" free-martins would seem to be excluded. The source of the ovarian sex hormone is by no means so certainly ascertained as that of the testicular hormone. The weight of opinion inclines to the view that the cells of origin in the ovary are derived from atretic follicles. If this view should be confirmed, the time of first formation of specific female sex hormones would be postponed in most species until after birth.

Doncaster's suggestion (1920) that the tortoise-shell tomcat may be a female transformed by hormones of a male partner in fœtal life has been dealt with by C. C. Little (1920, 1921), who finds no vascular connections between the membranes of fœtal cats. The suggestion, however, leads me to emphasize the finding that conditions in the free-martin, which offer probably the most favorable possible opportunity, do not lend themselves to the idea that complete sex inversion by hormones is a possible thing. I am led to emphasize this because Julian Huxley (1922, pp. 201 and 214) also states, basing his statement on the free-martin, that hormones may produce all stages of conversion of one sex into the other. Although I have paid particular attention to this point, I have been able to find no evidence for it. On the contrary, the conversion of the female sex in the case of the free-martin stops very far short of complete inversion. There is a wide gap between the most extreme case and the normal male, and the statistics do not favor the suggestion that in some cases this gap may be crossed at a bound, leaving no intermediates (cf. p. 50). I believe, however, that proper caution admonishes us to realize that the limits of hormone action may vary, even widely, in different species. Both Steinach and Moore, for instance, find considerable differences between the rat and guinea-pig in the feminizing effect of ovarian grafts on castrated infantile males. I do not, therefore, mean to deny the possibility of complete sex inversion by hormones for all species, but merely to emphasize that the case is not proved for any species.

So far it has not been shown that the explanation of the intersexual condition in the free-martin is of wider application for the explanation of intersexuality in mammals. The theories must, however, develop along two main lines, viz.: either that of unbalanced sex factors or of unbalanced hormones. The former is a problem in genetics, which will be advanced by actual breeding experiments, for which goats seem to offer unusual advantages owing to the relative abundance of hermaphrodites in certain breeds, and the negative evidence against the theory of unbalanced sex hormones in this case. The sex hormones may be unbalanced as against each other or as against genetic sex factors in other ways than by anastomosis of fœtal circulations in different-sexed individuals. As I pointed out previously, there may be a breaking down in the presumable defence mechanism of the placenta that protects the embryos from the sex hormones of the mother. Possibly other causes may also exist which in the present state of development of sex hormone theory can not be anticipated.

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APPENDIX.

Supplementary List of Cattle Twins Examined in Utero.

The list published previously included the first 57 cases. A comparable table of cases 58–92 is herewith given to make the record complete.

Supplementary List of Cattle Twins Examined in Utero.

No.	Sex.			Size.	Chorion.	Maternal Ovaries.
	♂	♀	☿			
58	2			14.5 cm. each.	Single.	Ovaries absent.
59		2		19.5 cm. each.	Single.	Ovaries absent.
60	1		1	♂ 16 cm., ☿ 15.2 cm.	Single.	One ovary present with corpus luteum.
61		2		23 cm., 24 cm.	Single.	Ovaries absent.
62	1		1	♂ 4 cm., ☿ 3.75cm.	Single.	One ovary present with corpus luteum.
63	1	1		22 cm. each.	Chorions separate.	Ovaries absent.
64	1	1		♂ 14 cm., ♀ 13.75 cm.	Chorions separate.	Both ovaries present. Corpus lut. in each.
65	1		1	♂ 17 cm., ☿ 16.75 cm.	Single.	Both ovaries present. Corpus lut. in each.
66	See Note 1.					
67		2		10 cm., 10.25 cm.	Single.	Ovaries absent.
68	2			9.5 cm., 10.3 cm.	Single.	Ovaries absent.
69	2			7.8 cm., 8.2 cm.	Single.	Both ovaries present. Corpus lut. in each.
70	2			13 cm. each.	Single.	One ovary present with corpus luteum.
71	2			17 cm., 18 cm.	Single. Narrow connection.	Both ovaries present. Corpus lut. in each.
72		2		28.5 cm. each.	Single. Both embryos in one horn of uterus.	Both ovaries present. 2 c. l. in one. None in other.
73	2			6 cm. each.	Single.	Both ovaries present. Corpus lut. in each.
74		2		12.5 cm., 13.5 cm.	Single.	Ovaries absent.
75	1		1	16 cm. each.	Single.	Ovaries absent.
76		2		15.5 cm., 16 cm.	Single.	Both ovaries present. Corpus lut. in each.
77	1		1	♂ 10 cm., ☿ 9.4 cm.	Single.	Ovaries absent.
78	1		1	32 cm. each.	Single. Both embryos in one horn of uterus.	Both ovaries present. Two corpora lutea in one. None in other.

Note 1.—Five days' old free-martin, twin to bull. Preserved material for histology. See Willier ('20).

No.	Sex.			Size.	Chorion.	Maternal Ovaries.
	♂	♀	☿			
79		2		26 cm., 27 cm.	Single.	One ovary present with corpus luteum.
80		2		16 cm. each.	Single. Both embryos in one horn of uterus.	Both ovaries present, but only one corpus luteum.
81	1		1	14 cm. each.	Single. Narrow connection.	Ovaries absent.
82	2			13 cm. each.	Single.	One ovary present with corpus luteum.
83		2		12 cm. each.	Single.	Both ovaries present. Corpus lut. in each.
84		2		15 cm. each.	Single.	Both ovaries present. Corpus lut. in each.
85	1		1	11.5 cm. each.	Single.	One ovary present with corpus luteum.
86		2		Note 2.		
87		2				
88		2				
89	2					
90	2					
91	1		1	15 cm. each.	Single.	Ovaries absent.
92	1		1	♂ 12 cm., ☿ 11.5 cm.	Single.	Ovaries absent.
93	1	1		♂ 10.25 cm., ♀ ♀ 7.75 cm.	Narrow non-vascular connection.	Both ovaries present. Corpus lut. in each.
94	1		1	♂ 19.25 cm., ☿ 19 (?) cm.	Single.	Both ovaries present. Corpus lut. in each.
95	2			4 and 5 cm.	Single without constriction. Both embryos in one horn.	Both ovaries present, two corpora lutea in one; none in the other.
96		2		Not recorded.		Absent.
97	1		1	About 15 cm. each.	Single, without constriction. Both embryos in one horn.	Absent.

Note 2.—Numbers 86 to 90 inclusive were collected during my absence in January and February, 1920; no records but sex were taken.

COPPER, ENZYMES, AND FERTILIZATION.

OTTO GLASER,

Amherst College.

I. Introduction.

The inhibition of development in *Arbacia* by infinitesimal quantities of copper salts has lately been employed by F. R. Lillie ($21^{1,2}$) as an expedient in studying the fertilization reaction. The results are highly suggestive and the novelty of the procedure altogether likely to inspire much further work. Yet even now there are complications to be dealt with before one can realize on the possibilities which apparently inhere in the method.

In the first place, there is the matter of effective concentration. This has two aspects: how much of the copper salt added to sea-water actually remains in solution and what proportion of the permanently soluble fraction reaches the eggs? Moreover, is the chorion involved? There is no reason for thinking that copper would escape concentration in the egg-jelly since, as I have recently shown (22^1) this is true of all the salts to which the egg is normally exposed. And, finally, is it possible for a marine organism to carry a "copper-avid substance in the cortex of the egg" and escape the all but inevitable consequence?

II. The Precipitation of Copper Sulphate in Sea-water.

The first step was a study of the solubility of copper sulphate in sea-water. Since my program involved reliance on chemical, rather than physiological, means of detection, I employed concentrations well within the sensitivity range of my first methods. I therefore prepared a standard solution of $CuSO_4 . 5H_2O$ in distilled water such that each cubic centimeter corresponded to one milligram of the original salt. This was equivalent to .276 mg. of metallic copper per c.c., or $Cu = m/230$ and $n/460$.

This standard was compared colorimetrically with a duplicate solution in sea-water. After correcting for the few drops of ammonia added in order to intensify the blue color, it was found in three closely concordant experiments that 36.9 per cent. of the copper had been precipitated in the sea-water—in all probability—as insoluble basic carbonates. The sea-water, therefore, retained in solution a trifle over .174 mg. Cu per c.c.—the equivalent of Cu $= m/365$ or $n/730$.

III. The Removal of Copper from Solution by Normal Eggs.

The precipitated fraction of the copper sulphate interferes seriously with attempts to measure colorimetrically the quantities actually removed by the eggs. Not only is the turbidity of the solution objectionable, but the precipitate adheres mechanically to the chorion, which in turn goes slowly into solution. It is absolutely essential to eliminate the copper carbonates.

However, a concentration of $n/730$, even after the carbonates are removed, often destroys the eggs in all except brief exposures. This difficulty can be overcome by dilution to $n/1{,}460$. At this concentration .2 c.c. of dry ripe eggs in 75 minutes reduced the copper content of 14.8 c.c. of solution as follows:

Lot *A* from $n/1{,}460$ to $n/1{,}810.4$.
Lot *B* from $n/1{,}460$ to $n/1{,}766.6$.
Lot *C* from $n/1{,}460$ to $n/1{,}785.5$.

Very definitely, then, *Arbacia* eggs are able to remove copper from solution, yet, despite their close agreement, the figures in reality have no quantitative standing. They completely mask an important source of error.

IV. The Rôle of the Chorion.

If the chorion takes part in lowering the concentration of copper solutions, eggs without jelly should lose at least part of their effectiveness. But to demonstrate this is not easy. The very removal of the chorion, of itself, introduces variables which, in the present case at least, might be increased in number or aggravated by the use of dilute hydrochloric acid as a solvent of the jelly. This restricts us to the mechanical methods with their relative violence and uncertainty. One can never feel confident that every egg has lost its chorion, nor that the shaking, flipping—or whatever one does—leaves every egg intact. Some control of these errors is possible by microscopic examination in suspensions of india ink, and, as a matter of fact, I never employed material unless the eggs appeared uninjured, nor unless at least 90 per cent. failed to reveal the jelly. However, it was impossible to offset the fact that equal volumes of normal and dechorionized eggs can not contain equal numbers. It is not surprising, therefore, that comparisons with normal material should prove variable; nevertheless, in view of my other experiments, I did not expect to encounter the irregularities actually found. These were so great that the results are best appreciated in the following form:

	Excess Copper Removed in 75 minutes from 10 cc. *n*/1460 Solution by	
	.2 cc. Normal Eggs	.2 cc. Dechorionized Eggs
Lot A	23.3%	
Lot B		11.5%
Lot C	29.5%	
Lot D	4.0%	
Lot E		2.0%
Lot F		8.3%
Lot G	13.7%	
Lot H	54.0%	
Ratio	5.7	: 1

This clearly does not indicate that dechorionized eggs remove no copper from solution, but that normal eggs, on the average, remove more. If we neglect the numerical differences in equal volumes of the two classes of eggs—a handicap distinctly against the normal material—we can say that the copper profit of normal was to that of dechorionized eggs at least as 5.7 : 1.

It follows that the chorion is heavily involved in the removal of copper from solution. Yet the amounts taken up can not be determined even from my original values; nor can these be used as a basis for predicting the precise outcome of any particular competition for copper between normal and dechorionized eggs. Indeed, the results, though valid enough, once more mask a fundamental source of error.

V. Irregularities in the Removal of Copper from Solution.

The irregularities encountered were of several sorts. Equal quantities of eggs, during equal exposures, did not invariably appear to remove equal quantities of copper from a *n*/1,460 solution. In fact, in certain instances, the solutions after exposure to the eggs seemed to contain more copper than I had added. With dechorionized material the results were almost bizarre. Such eggs usually appeared to absorb less than the normal, though at times the opposite was true. Their most frequent irregularity was the apparent addition of copper to the sea-water. Where was the error?

I sharpened my technique as much as possible. After each experiment a few drops of sulphuric acid were added to the solution to be tested in order that any copper carbonates formed during the experiments might be converted. I also used ammonia to intensify the colors. Nevertheless the harvest of inconsistencies continued.

It was then that I recalled Lillie's "copper-avid substance in the cortex of the egg." This might explain everything, yet only if it could be shown that the untreated normal *Arbacia* egg actually contains copper.

VI. Demonstration of Copper in *Arbacia* Eggs.

A. Preliminary Considerations.

Was there any likelihood of demonstrating copper in the normal egg? In the literature I found the following especially pertinent facts:

100 gr. fresh *Asterias rubens* contain 2.45 mg. Cu.
100 gr. fresh *Stichopus regalis* contain 2.83 mg. Cu.
100 Sepia eggs contain 0.5–0.8 mg. Cu.
100 gr. dry hen's yolk contain 2.00 mg. Cu.

The first three items are from Aron's ('09) discussion in Oppenheimer's Handbuch; the last from an investigation by Fleurent and Levi ('10^1).

Measurable quantities of copper, then, have been reported for certain echinoderms and at least two kinds of eggs.

B. Qualitative Tests for Copper.

It is hardly necessary to discuss the very large number of preliminary tests made at Woods Hole during the summer of 1921.

It is sufficient to say that whenever I was able to avoid interference, due to small amounts of iron, I found positive indications for traces of copper. Material was, therefore, prepared for the more careful analyses made this winter at Amherst.

Immature eggs were taken directly from the ovaries; mature eggs were shed spontaneously under conditions which I have described elsewhere (22[1]) and which precluded any contact whatever with sea-water, fresh water, dermal secretions, appendages, or detritus of any kind. The eggs were tested with sperm and found to be normal.

Two c.c. of mature eggs dissolved in concentrated nitric acid constitute sample *A*. This was subsequently diluted to 50 c.c. with water doubly distilled from glass. Fractions of this material were used either directly or, after evaporation to dryness and incineration in glazed china crucibles. The ash was redissolved in sulphuric acid, usually neutralized with ammonia, and the volume restored to that of the original fraction. In all these operations great care is essential. All reagents were tested repeatedly and found negative for copper.

1. *Copper as Cupric Hydroxide*—$Cu(OH)_2$.

   1 c.c. of *A* evaporated to dryness and incinerated. Ash dissolved in H_2SO_4. Excess NH_4OH should give the blue of cupric hydroxide. Result: positive but exceedingly faint.

2. *Copper as Cupric Cyanide*—$Cu(CN)_2$.

   To 5 c.c. of *A*, added concentrated KCN solution. Should give yellow precipitate of $Cu(CN)_2$. The precipitate should dissolve in H_2SO_4 and this solution should give test for $Cu(OH)_2$. Result: positive and distinct throughout.

3. *Copper as Cupric Ferrocyanide*—$Cu_2Fe(CN)_6$.

   Ash from 10 c.c. of *A* redissolved and neutralized as before. To avoid interference by iron, the reagent, a 1:35 solution of potassium ferrocyanide, $K_4Fe\ (CN)_6$ in distilled water, was carefully run over the surface of the test solution as in the ring reaction for proteins with nitric acid. In the present case also a ring is formed; this quickly thickens—the upper layers being composed of the blue ferrocyanide of iron; the lower of brown cupric ferrocyanide.

4. *Copper as Cupric Xanthate*—$Cu(C_3H_5S_2O)_2$.

   Redissolved ash from *A*, in the presence of potassium ethyl xanthate should give a yellow color due to the formation of copper xanthate

The test which is sensitive roughly to 1 part in 50 million, is not vitiated by the presence of sea-salts nor by small amounts of iron, lead, nickel, cobalt, zinc, or manganese. Results: Positive and very decided.

5. *Copper as Crystalline Cupric Sulphate*—$CuSO_4 \cdot 5H_2O$.

After precipitation of the iron as hydroxide the ash was redissolved in sulphuric acid and drops of the solution allowed to evaporate to dryness on a slide. Under the microscope long blue needles corresponding to copper sulphate. The blue color greatly intensified by ammonia.

6. *Copper as Metallic Copper*—Cu.

5 c.c. of *A* plus excess NH_4OH. Copper if present in sufficient quantity should be deposited on tin-foil. Result: positive but faint. With aluminum, however, despite incompleteness, the deposition was very marked; film, rose-colored by reflected light; bright and typically copper-colored when polished.

7. *Copper by Electrolytic Deposition.*

10 c.c. of *A*. Platinum Electrodes. Voltage 2.6; amperage 1/10. At this tension copper and only copper can be deposited on the kathode. After 24 hours, result positive and distinct. Could not be weighed. Redissolved deposit and got positive test with potassium ethyl xanthate.

On the basis of this evidence it was no longer possible to doubt the existence of copper in the normal *Arbacia* egg. The basic necessity for a secretion of copper compounds or copper-bearing substances is therefore given. If such materials are held up temporarily by the chorion, the differences between normal and dechorionized ova, noted in sections IV. and V., would be explained. But do the eggs really secrete copper in any form? We could perhaps answer this question if we could associate the metal with definite structures and substances in the egg.

VII. Localization of Copper in the *Arbacia* Egg.

The problem calls for the localization of the copper. I employed direct methods as well as indirect. The eggs used were shed under the conditions previously mentioned and fixed in absolute alcohol. I studied both whole mounts and sections, 5 μ in thickness. The latter, after removal of the paraffin, like the unsectioned eggs, were treated with various reagents and examined in the opaque condition, wet or dry; or after clearing, as transparent objects, in glycerine or balsam. All the reagents were tested and again found negative for copper.

1. *Copper in the Egg Pigment.*

Since copper is an integral part of so many organic coloring matters, it was natural to examine first of all the pigment of the egg. This material, as is well known, is widely distributed in granular form in the cytoplasm. As the granules are most numerous in tangential sections, the pigment bodies are evidently concentrated near the surface. This also seems to be true, to some extent, immediately about the nucleus.

It is entirely justifiable to identify these granules as the source of the coloring matter secreted by the eggs. I consequently precipitated the pigment from normal egg secretion by the chloroform method described in an earlier paper (21^3). After incineration an alkaline solution of the ash in twenty minutes gave a relatively heavy, though incomplete, deposition of metallic copper on aluminum. The deposit was dissolved and gave a deep yellow color on addition of potassium ethyl xanthate.

Direct proof of copper in the pigment bodies *in situ* is also possible. Tangential sections which contain more of these granules than others are the very ones that stain most heavily with hæmatoxylin. This we should expect in the presence of copper, though the test is not dependable if iron is also present. I therefore adopted a procedure employed in the microchemical analysis of minerals. The method depends on the formation of a triple nitrite of potassium, copper, and lead ('94).

To sections on the slide I added a trace of sodium acetate and a somewhat larger quantity of potassium nitrite. The whole was then acidulated with acetic acid. Last of all, a few grains of lead acetate. The presence of copper, as little as 0.05 microgram,[1] if crystals are desired, completes the conditions necessary for the formation of the triple salt, $K_2CuPb(NO_2)_6$, which is jet black.

In this test, which differentiates between iron and copper, the sections most darkened are the very kind most affected by hæmatoxylin. There is a marked graying of the cell contents to which a partial reduction of the pigment bodies contributes. The general effect is due to a decided increase in cytoplasmic granulation—the granules being excessively minute and, like the microsomes of the cytologists, black. If the material is kept moderately warm on a water bath and the reaction is permitted to continue for half an hour, the pigment bodies themselves become distinctly bluish or even black. This suggests a gradual unmasking of the copper in Macallum's sense ('12).

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[1] 1 microgram (μgr.) = 1/1000th milligram.

2. *Copper in the Egg Membrane.*

A definite visible membrane ('13[1]) invests the unfertilized *Arbacia* egg and constitutes a barrier through which pigment must pass in order to reach the outside. If now the pigment itself contains copper, the same thing might easily be true of the membrane through which it passes.

But the membrane is by nature yellowish-brown. This excludes tests depending on the formation of cupric cyanide, ferrocyanide, or xanthate. With hæmatoxylin a bluish tint develops, and this could be interpreted as evidence of copper if iron were absent. However, since the latter is almost certainly present, we must fall back on the triple-nitrite reaction.

In this the original native yellow-brown of the membrane is replaced with black. Under low powers the membranes are in very sharp relief. The oil immersion, however, resolves their uniform black, in optical section, into a series of irregularly spaced discontinuous beads connected by an exceedingly thin continuous black line.

This test was repeated on several sets of sections. The results were uniform and without inconsistencies. The evidence, then, that copper exists in the *Arbacia* egg membrane appears valid; yet the quantities involved are so small that I felt impelled to check the results on the hen's egg, whose vitelline membrane, I reasoned, should contain copper if the analysis of the yolk by Fleurent and Levi (*loc. cit.*) is correct.

This supposition was readily substantiated by four different methods.

The vitelline membranes were removed from the yolk and washed in a stream of running tap-water for twelve hours. In this way all but negligible quantities of yolk were removed.

After incineration a solution of the ash, upon the addition of ammonia, turned blue. This test was supported in neutral solution by a marked yellow brought about by potassium ethyl xanthate. Again the triple-nitrite test was positive. Under the microscope the treated membranes showed numerous irregular blotches and specks of black. By prolonging this treatment for thirty minutes and following it with a short exposure to 100° C., the membranes became closely speckled with black and underwent a discoloration quantitatively more in harmony with the very marked reaction gotten with ammonia and potassium ethyl xanthate. The copper in this membrane is, therefore, also "masked"; moreover, the discovery of copper in the *Arbacia* egg membrane does not stand alone.

3. *Copper in the Chorion.*

Since the pigment during its outward passage permeates the chorion, the latter necessarily contains traces of copper. There is also some direct evidence of this, for hæmatoxylin imparts to the jelly a light bluish tint, whereas the triple-nitrite test brings out a few scattered granules stained black. Infinitesimal quantities of copper, then, can be demonstrated directly in the chorion.

4. *Copper and the Cortex.*

Inasmuch as Lillie (*loc. cit.*) has assumed the presence of a copper-avid substance in the cortex of the egg, I paid especial attention to this region both in untreated sections and in those exposed to reagents for copper.

Unquestionably the cortex differs from the remainder of the egg. Yolk, pigment, and other visible granules are absent. The zone immediately beneath the egg membrane, even at very high magnifications, seems to be optically clear. In sections not exposed to reagents for copper the cortical layer appears blue—no doubt the result chiefly of refraction. Nevertheless the color is intensified by ammonia and hæmatoxylin and does not disappear entirely when viewed by light transmitted through ground glass nor after the sections have been cleared in glycerine, xylol, or balsam.[2]

Furthermore, perhaps on account of distortions through shrinkage, the blue layer, instead of being in immediate contact with the membrane, not infrequently lies at a considerable distance beneath it. All this is indicative of a region differentiated from the remainder of the egg.

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[2] There are, of course, other effects of refraction throughout the egg.

Is copper demonstrable in this layer? None of the direct methods could give an unequivocal answer, though the triple-nitrite test did reveal a few black granules similar to the black beads found in the membrane. This might be considered a demonstration of copper if the displacement of an occasional pigment granule in the process of sectioning could be altogether set aside. But there are other considerations.

The fertilization of egg fragments has been reported by numerous investigators, including myself ('13[1]), yet the correctness of these observations has been doubted because some egg fragments are incapable of fertilization. The difference is accounted for in the work of Chambers,[3] who finds that fragments derived exclusively from the interior of the egg can not be fertilized, whereas those containing a fair portion of the cortex respond like the original egg. This evidence, if not compelling, at least indicates more immediately than any other the physiological distinctiveness of the cortex and the necessity of cortical materials in fertilization.

Now, certain secretions of the egg are likewise necessary. This was first shown by F. R. Lillie ('13[2]), and subsequently by my sterilizations with charcoal ('21[4]). A logical combination of our two necessities traces the secretions to the cortex. But are they—or perhaps better, their forerunners—really there? For the normal egg this has not been demonstrated, nor is the cortex, in all probability, their only location; for egg fragments, with chorion and membrane both removed, the inference appears less hazardous. The concentration, then, of the secretions, or their forerunners, in the cortex of the egg may be assumed as a not unreasonable working hypothesis.

On the basis of isolable precipitates, Miss Woodward ('18) and I ('21[5]) have contended that these secretions contain at least two separate substances—a lipolytic ferment and a material which agglutinates spermatozoa. The immediate problem consequently narrows down to this: Is copper demonstrable in precipitated lipolysin and agglutinin?

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[3] See Lillie's "Problems of Fertilization," p. 264.

A. *Lipolysin—I.*

The lipolysin first tested was prepared as follows: the eggs were permitted to secrete as usual; the agglutinin was salted out with ammonium sulphate, which incidentally also precipitates much of the pigment. After filtration, the lipolysin iteslf was thrown down by means of barium, redissolved in dilute hydrochloric acid and precipitated by acetone which unless in great excess holds the remaining agglutinin in solution. The lipolysin so secured was a white powder free from pigment, though in the course of time it turned slightly purple.

After incineration a solution of the ash gave a positive reaction for copper with potassium ethyl xanthate.

Lipolysin—II.

This precipitate had exactly the same history as the foregoing except that charcoal was used in place of barium. Moreover, the powder did not change color but remained snow white and therefore may have been a somewhat purer product than lot I. By the xanthate test, lot II. also contained copper.

B. *Agglutinin—I.*

The agglutinin first examined came from *Asterias* egg-secretion which had been freed from lipolysin by the barium method. The xanthate test, carried out as before, gave a perceptible reaction for copper but as the separation from the other constituents of the secretion was incomplete, this result is inconclusive.

Agglutinin—II.

A second test was made, this time with *Arbacia* agglutinin which had been merely salted out with ammonium sulphate and contained therefore considerable quantities of pigment. In this case the xanthate test was markedly positive.

Agglutinin—III.

Arbacia agglutinin whose separation from pigment and other impurities was accomplished by chloroform, charcoal, and final differential precipitation from acetone. This material was negative for copper.

In all I examined five different lots of agglutinin, and in every case preparations whose purity I supposed to be high were the ones that proved to be either completely negative for copper, doubtful, or at most very slightly positive. It appears that copper is not an essential constituent or even regular associate of pure agglutinin.

These results, together with the triple-nitrite test and the considerations that suggest a cortical concentration of the secretions, justify my assumption that copper is present in the cortex of the egg immediately beneath the vitelline membrane. This copper, however, is not associated with the egg pigment or the agglutinating material, but with the lipolysin, whose concentration in the cortex is thus one step nearer to being a demonstrated fact.

VIII. Copper and Enzymes.

Lipolysin is or contains a lipolytic ferment whose presence accelerates the hydrolysis of higher and lower fats ('22[2]) and the synthesis of ethyl-butyrate ('22[3]). The association of copper with this enzyme, though contrary to expectation, can not be evaded. Does the case stand alone?

Apparently not. For example, S. Yagi ('10[2]) finds a thirty-four fold increase in the copper content of the rabbit's liver when the animal is fed 4.8 gr. copper sulphate in assimilable doses during a period of seventeen days. This agrees perfectly with the results of Titze and Wedemann ('11) on the goat and those of Rose and Bodansky ('20[1]), who find the copper content of the oyster's hepato-pancreas to be twice that of the muscle. Although as yet no one has risked the suggestion, it seems apparent that copper concentrates in tissues rich in lipases and certain other enzymes. May we risk another step and say the copper is concentrated in the enzymes? Be this as it may, it was these considerations that led me to test the following commercial preparations:

A. *Pancreatin* (Merck's).

This preparation contained all the pancreatic enzymes. The ash from about .2 gr. gave a marked reaction for copper as copper xanthate.

B. *Pancreatin from Pig* (Squibb's).

Result exactly as in *A*.

C. *Pancreatin* (Parke-Davis).

Result as in *B* and *A*.

Is the copper associated with the lipases exclusively? This can not be determined from the above tests. I therefore tried a proteolytic enzyme likely to be free from lipases, amylases, and other enzymes.

D. *Pepsin* (origin unknown).

The ash from .2 gr. when freed from iron gave no positive indication for copper. However, the iron hydroxide was found to hold the copper back. I therefore washed the precipitated hydroxide with ammonia, whereupon the xanthate gave a very faint though unmistakably positive test.

E. Pepsin (Parke-Davis).

Also positive; faint after precipitation of copper by iron hydroxide.

These tests prove that copper occurs in preparations of pancreatic enzymes and pepsin. The association with lipolysin, therefore, is not a necessarily isolated instance, but very possibly a special illustration of a general rule. Indeed, the studies of von Euler and Svanberg ('20^2) definitely foreshadow a natural relation between enzymes and heavy metals.

IX. Localization of Copper in Eggs Exposed to Copper Solutions.

Normal eggs, in addition to secreting copper compounds, remove copper sulphate from sea-water when this salt is present. In eggs so exposed we should expect differences, if not in distribution, then at least in the concentration of the copper which they contain. Such analyses seem doubly worth while, for they seem not only to control and correct the observations on normal material, but may ultimately throw some light on the machinery of copper fixation and metabolism in general.

Tests consequently were made on material with a total volume of .5 c.c. exposed for 20 minutes to 5 c.c. of a 1/180 *n* Cu solution in sea-water. In every other respect the eggs were treated as described in section VII. Moreover, the material was from the same lot as that used for the localization of copper in normal eggs.

1. *Copper Absorbed by the Chorion.*

From the experiments of section IV. we expect for the jelly of exposed eggs a copper content greater than that found in the normal chorion. This expectation, of course, is realized, as can be shown by means of the hæmatoxylin test with entire eggs. Superficially such eggs take on an unmistakable blue color which microscopic examination localizes distinctly in the jelly. The same effect is gotten with the triple-nitrite test, only the color is then black instead of blue.

2. *Copper Absorbed by the Egg Membrane.*

With hæmatoxylin the membrane of the exposed egg is bluer than the normal. The triple-nitrite test, however, is more convincing. In optical sections of the surface one no longer finds individual black beads strung on a thin thread, but a heavy continuous black line. Even the highest powers fail to resolve the continuity. It seems very clear, indeed, that potassium-copper-lead nitrite is more abundant than in normal membranes.

On this basis it is possible to understand one of Lillie's results. If an excess of copper in the membrane alters it so that spermatozoa are not able to penetrate as easily as under normal conditions, we should expect "a certain virtue in mass action in the presence of this inhibitor of fertilization" (*loc. cit.,* p. 129). I noted exactly the same virtue ('15) in connection with eggs which had been exposed to calcium. This particular point and its meaning have both been overlooked in subsequent discussions.

3. *Copper Absorbed by the Cytoplasm.*

Inside the exposed egg there are differences in the same sense. The number of black granules after the nitrite treatment is greatly increased. As a whole and in sections the exposed egg is distinctly darker than the normal. Copper evidently passes inward through the membrane and diffuses generally through the cytoplasm.

4. *Copper Absorbed by the Cortex.*

If, as Lillie's experiments indicate, the cortex contains "unsaturated" copper-avid material, one may expect very considerable differences in the cortical zone. As a matter of fact, the triple-nitrite test brought to light black beads similar to those seen in and immediately under the normal membrane; they were decidedly more numerous than in normal eggs and gained in distinctness by heating. Furthermore, the blue zone which in normal eggs resists resolution by all the methods employed now differentiates slightly with both potassium ethyl xanthate and potassium ferrocyanide. With the first, the blue acquires a greenish tint and becomes separated from the egg membrane by an exceedingly narrow yet distinguishable yellowish layer; with the second, a very thin brown band intervenes between the original blue and the membrane.

These changes become intelligible if we assume, in the one case, the formation of minute traces of copper xanthate; in the second, of copper ferrocyanide. Since the same reagents produce no noticeable changes in these locations in untreated material, it appears that the cortex of an egg exposed to copper contains more than the normal quantity of the metal. Moreover, the excess is localized chiefly near the surface, and since it is not the agglutinin that is copper bearing, but the lipolysin, it appears probable that the latter is also concentrated immediately under the vitelline membrane.

X. Copper Map of the Normal Egg.

From all these tests—those on the controls as well as those on material exposed to copper sulphate, I have constructed a chart which indicates the distribution of copper in the normal *Arbacia* egg. This map is a visual summary of the chief results and inferences, and in view of the preceding discussion seems to require no other comment than that given in the legend under the figure.

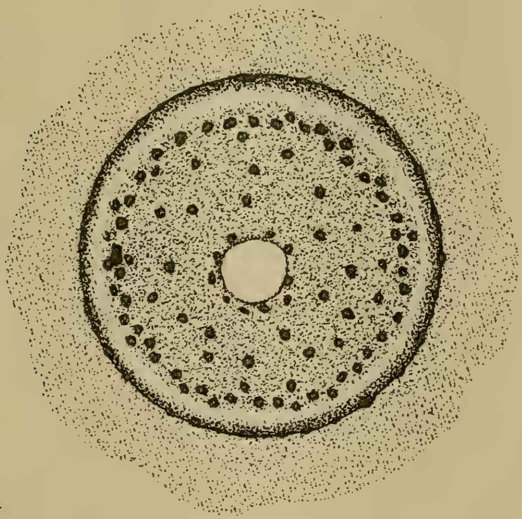

Diagram showing distribution of copper in the normal egg of *Arbacia punctulata*. The central clear area is the nucleus. Immediately about this, and extending to the cortex, the larger black spots represent pigment granules in which copper was demonstrated indirectly by analysis of secreted pigment and directly, in situ, as potassium-copper-lead nitrite. The latter, as well as hæmatoxylin, indicates also the presence of diffuse copper in the cytoplasm. This is shown by the finer stipples.

The cortical layer is shown as differentiated into two zones: an inner, free from copper; an outer, immediately under the egg membrane with considerable concentration of copper. The evidence is again the triple-nitrite test on normal eggs, and the xanthate and ferrocyanide tests on eggs which had been exposed to solutions of copper in sea-water.

The vitelline membrane is shown as a line whose irregularly spaced black beads represent regions in which the triple nitrite was concentrated. The membranes of eggs exposed to copper solutions appear as continuous heavy black lines without beads.

The region outside the vitelline membrane represents the chorion with its diffuse and highly rarefied copper.

The diagram is intended to indicate distribution. It cannot indicate quantitative differences accurately because a variety of tests was used and the extent of discoloration depends not primarily on the quantities of copper but on the molecular size of the copper compounds formed. These are necessarily different in the several tests.

XI. ORDER OF MAGNITUDE OF THE QUANTITIES OF COPPER INVOLVED.

Quantitative data on the copper content can not be given. The methods for determining the copper are, indeed, reliable enough; but, unfortunately, the chorion alone prevents anyone from knowing how many eggs are present in 1 c.c., and until this number is definitely ascertained our initial measurements can hardly articulate closely with the results of chemical methods unusual in delicacy. For the present, then, we can arrive only at approximations.

1. *The Ovarian Egg.*

Eleven c.c. of ovary were dissolved in 11 c.c. of concentrated nitric acid and after complete destruction diluted to 100 c.c. with distilled water. Ten c.c. of this solution were evaporated to dryness and the incinerated ash dissolved. After neutralization with ammonia the volume was restored to 9 c.c. To this was added 1 c.c. of a solution of potassium ethyl xanthate whose concentration was 1 gr. per 1,000 c.c. of distilled water. This same amount was added to 9 c.c. of a copper sulphate solution containing 10 μ gr. of copper per c.c.

Colorimetric comparisons were then made, and the concentration, C_2, of the unknown calculated from the concentration, C_1, of the standard and the depth of the unknown, D_2, necessary to match a given depth, D_1, of the standard. Accordingly,

$$C_2 = \frac{C_1 \times D_1}{D_2}.$$

Test	$\frac{C_1 \times D_1}{D_2}$	Micrograms Cu. per c.c. Eggs.
I	$\frac{10 \times .50}{2.21}$	20.54
I	$\frac{10 \times .30}{1.38}$	19.76
II	$\frac{10 \times 2}{1.40}$	14.29
II	$\frac{10 \times 2}{1.31}$	15.27
III	$\frac{10 \times 1.5}{1.12}$	13.39
		Average = 16.65 μgr.

2. *The Shed Egg.*

Ten c.c. of the material referred to as *A* in section VI. were treated and tested in exactly the same way.

Test	$\frac{C_1 \times D_1}{D_2}$	Micrograms Cu. per c.c. Eggs.
I	$\frac{10 \times 1}{1.55}$	161.25
I	$\frac{10 \times 1.5}{2.2}$	170.50
II	$\frac{10 \times 1}{1.40}$	178.50
II	$\frac{10 \times 1}{1.33}$	188.00
		Average = 174.56 μgr.

3. *The Fertilized Egg.*

Forty c.c. from a solution of 1.2 c.c. of fertilized eggs gave the following results:

Test	$\frac{C_1 \times D_1}{D_2}$	Micrograms Cu. per c.c. Eggs.
I	$\frac{10 \times .5}{2.00}$	20.83
I	$\frac{10 \times .5}{1.96}$	21.25
I	$\frac{10 \times .5}{2.03}$	20.50

Average = 20.86 μgr.

The copper content of the fertilized ovum apparently has the general order of magnitude characteristic of the unshed ovarian egg. That of the shed egg is, roughly, from eight to ten times greater.

XII. Discussion.

1. *The Copper Problem.*

Unless bound up in respiratory or other pigments, copper is considered essentially a poison. Its use in germicides and fungicidal solutions, the harmful influences on higher and lower plants, power to block organic catalysis, the medicinal properties of colloidal suspensions and copper salves—all support the prevalent view. Yet there are no poisons in nature; there are only poisonous effects. These may be exercised by the commonest articles of diet at certain concentrations and by copper, it happens, at very low ones.

Even a cursory examination of the literature suggests further misgivings. Copper has been found repeatedly in three of the five main divisions of the plant kingdom and among animals, with equal frequency, in nine phyla, ranging from protozoa—if *Volvox* ('21[6]) is an animal—to man. However, only Maquenne and Demoussy ('20[3]), who worked on plants, and Rose and Bodansky ('20[1]), whose studies cover a wide range of marine animals, are actually bold enough to suggest that copper in general may be more than an adventitious element which living things somehow tolerate.

Specific processes in which it normally plays a rôle are still unknown; moreover, they are quite likely to remain so unless we free ourselves for investigation by an admission of ignorance.

As a matter of fact, copper has many of the qualifications of a biological element. It is widely diffused in the environment and has been so since earliest geological time. Though listed among the less active metals, it is capable of entering into a large variety of combinations, including numerous organic unions. It is difficult to see how a living thing could avoid copper except by some definite mechanism of exclusion.

The absence of this is significant, but does not establish physiological importance. Very possibly living things are mere sieves that hold the copper back; very possibly, too, its marked concentration in such tissues as the liver is a liability, by accident dependent on other attributes and under ordinary circumstances negligible. Still one would like to be certain, and in our present state of knowledge this is impossible. Yet a negative answer, excluding copper from the realm of physiological processes, even now appears unlikely. It is impossible to conceive the synthesis of respiratory pigments, turacin, or any other product without thinking of a long linkage of reactions inevitably affected, directly or otherwise, by their final end result.

2. *Copper and Enzymes.*

Von Euler and Svanberg (*loc. cit.*) have shown that the addition of saccharase results in a marked reduction of the free silver ions in dilute solutions of silver nitrate. Since the silver did not become colloidal under the influence of reductions, possibly due to certain constituents of the enzyme preparation, these writers suggest a binding of the silver ions to certain constituents of the saccharase solution.

Since such unions render the enzyme inactive, why not assume that the constituent of the solution with which the metallic ions combine is the enzyme itself, an organic co-enzyme, or both? Such an assumption seems all the more reasonable because prolonged dialysis, I find, does not remove all the copper. Thus if copper incapacitates at all, catalysis would be excluded in any case.[4]

[4] It is not possible that certain differences of proportionality between the effects of silver and mercury salts could be explained on this basis? Perhaps too the recovery known as "The Danysz Phenomenon" results from a redistribution of metallic ions between enzyme and co-enzyme. Even if co-enzyme and enzyme together make up, and are identical with, the entity, enzyme, the allocation of Cu to different positions in this system might have results essentially those suggested both in sense and in degree.

This assumption involves on the part of enzymes or organic co-enzymes a capacity for combining with heavy metals. Since this power is not only very great, to judge by the dilutions that incapacitate, but likewise inseparable from normal ferments, we should not be astonished if enzymes from copper-bearing organisms or tissues give positive results when tested for copper.

3. *The Oligodynamic Effects of Copper.*

The poisonous effects of extraordinarily high dilutions of metallic copper were first noticed by Nägeli ('93), who found one part in seventy-seven millions rapidly fatal to *Spirogyra.* Because of the extremely small quantities involved Nägeli spoke of "oligodynamic action."

As these results have been repeatedly verified on other forms, it may be convenient to retain Nägeli's term. However, "oligodynamic" need imply nothing more mystical than a chemical or perhaps physical relation between extremely small quantities of material. As yet no acceptable explanation has been offered.[5]

But there have been suggestions. Nägeli was able to destroy the toxicity of oligodynamic solutions by means of paper, wool, paraffin, gums, and gelatin. The experience is quite comparable to the protective action of egg-water, gum arabic, and gelatin described by Lillie ('21^2). Inasmuch as the copper in the first case at least is probably present as electropositive colloidal hydroxide or carbonate, Bayliss ('15^2) imagines adsorption on the electronegative surfaces of Nägeli's detoxicants as the basis of their antitoxic properties. Conversely, the toxic effects become explicable as the outcome of adsorption by electronegative surfaces or particles critically involved in or on the affected tissues.

At this point Bayliss leaves the problem. We still do not know why such minute quantities are effective. Do they cause precipitation? If so, how much precipitation can they conceivably bring about? And, finally, what is precipitated? The neutralization of electrical charges in all likelihood is unavoidably associated with the ultimate mechanism of oligodynamic activity. For the present, however, our concern must be with facts a trifle more immediate. From these, it seems to me, we can derive a second suggestion.

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[5] Quite recently, Saxl, as quoted by von Euler and Svanberg, assumed "dass die Metalle eine eigenartige Keimtötende Kraft besitzen, welche nicht mit der Löslichkeit der Metalle zusammen hängen soll." Saxl refers to a physical energy "die sich zunächst auf der Oberfläche der Metalle abspielt, jedoch auch in andere Medien übergehen kann" ('20^2 p. 378).

The catalytic power of colloidal platinum is destroyed by traces of hydrocyanic acid and can be restored by simple aëration. Moore ('21[7]) compares this experience of Bredig and his pupils with the effects of hydrocyanic acid on the peroxidases of the enzymic oxidation system. "If these peroxidases are responsible in tissue cells for the uptake of oxygen by the protoplasm, it may well be that the poisonous action of hydrocyanic acid in such minute doses is due to interference with the action of the peroxidases" (*loc. cit.*, p. 231).

Disregarding the specific case which Moore had in mind, there is implicit here a definite relationship between enzymes and oligodynamic action. This relation, however, is also implicit in the copper inhibitions of Lillie. Again the union of saccharase with silver or copper implied by von Euler and Svanberg, together with the natural occurrence of copper in preparations of lipolysin, pancreatic enzymes, and pepsin, now reported, I believe, for the first time, are just what one might expect if the oligodynamic properties of heavy metals are traceable to the activation or inactivation of enzymes. These effects must depend on the capacity of enzymes to bind the metal. Until stoichiometrical determinations have been made nothing further can be said about such unions. But no matter what their nature may be, their occurrence should result in the presence of heavy metals in enzyme preparations whenever such presence is a physical possibility.

But the presence of copper raises an apparent difficulty. One might arrive at the paradox that enzymes, by the very nature of the case, must be normally incapacitated. This absurdity is easily dispelled. There is no reason for considering the normal enzyme as saturated with copper. For that matter, the enzyme proper may not be involved at all, for the observed effects can all be explained equally well if the copper is held by organic co-enzymes where these are present. But in this case also we are not compelled to consider the co-enzymes as saturated.

On this basis it is possible to explain the most puzzling feature of oligodynamic action. It is not that copper, silver, gold, or any other metals have this or that effect, but that infinitesimal amounts give results apparently out of all proportion to the quantities involved.

In the case of copper, for example, I imagine the oligodynamic effect as due to the inactivation of that fraction of the enzyme or co-enzyme which was not inactivated by the copper present in the first place. Where the oligodynamic effect is produced by silver or some other metal, the total of inactivated enzyme or co-enzyme would be composed of two fractions—the one inactivated by copper, the other by silver.

This view of the case has stoichiometrical implications which further work may or may not justify. But however this may turn out, the presence and effect of copper in preparations of normal enzymes calls urgently for further study.

Quite apart from the biochemical questions that suggest themselves at once, the discovery carries with it problems of wide biological significance. Is the liability to inactivation compensated by the production of larger quantities of the enzymes? Or does compensation come about by variety and differential susceptibilities? Why the tremendous number of enzymes when, aside from a few cases of molecular rearrangement, the only radical processes we have to deal with are the reversible oxidations and hydrolyses?

4. *Copper and Fertilization.*

Probably most of the copper present in the *Arbacia* egg is incorporated in the pigment whose elimination discolors the sea-water. As I have shown ('14), the rate of pigment secretion is increased 50 per cent. while the eggs are undergoing fertilization. This explains why the copper content of fertilized eggs is so much lower than that of unfertilized. My values for the copper content indicate in fertilized eggs an order of magnitude quite different from that in ripe eggs and essentially the same as that of the immature ovarian ovum. Tentatively, therefore, one may hazard that, among other things, fertilization restores the copper content to an order of magnitude characteristic of the unripe egg.

In the absence of more accurate data it is premature to discuss this fact at length. Nevertheless we may possibly find along these lines some help in clearing up the uncertainties that now beset us. Very possibly the concentration of copper normally has something to do with checking the growth of the egg, whereas the heightened rate of secretion during fertilization restores conditions essential for further growth and development. No doubt the linkage can be pictured in several ways, but one way is this: the elimination of pigment from the egg might result in the production of fresh pigment, or some other product, which, if copper-avid, might also draw upon the copper of the enzymes and thus assist in the process of activation.

All this, however, is merely in the realm of possibilities. At the rate at which discoveries are being made in this field hardly anyone would wish to formulate a theory of fertilization. The process is far more complicated than it seemed ten years ago, and if one thing is more certain than another, it is that the major classes of evidence have not yet been handed in.

XIII. Summary.

1. Nearly 37 per cent. of the copper sulphate added to sea-water is precipitated at once when the concentration is $Cu = n/460$.

2. Two tenths c.c. normal *Arbacia* eggs in 75 minutes reduce the concentration of 14.8 c.c. of a Cu solution from $n/1{,}460$ to $n/1{,}790.9$.

3. In this reduction the egg jelly or chorion is heavily involved.

4. It was impossible to determine the quantities of copper absorbed by normal eggs and eggs without jelly because of an apparent secretion of copper by the eggs themselves.

5. Therefore, the demonstration of copper in *Arbacia* eggs was undertaken. The copper was identified as cupric hydroxide, cupric cyanide, cupric ferrocyanide, copper xanthate, crystalline cupric sulphate, as metallic copper on tin-foil and aluminum, and finally by electrolytic precipitation under conditions under which copper and only copper could be deposited.

6. The copper was localized in the egg directly by means of hæmatoxylin and the triple-nitrite of potassium-copper and lead. Indirectly it was localized by the analysis of egg secretions.

7. The copper occurs chiefly in the egg pigment; the vitelline

membrane and the chorion also contain copper. It is not a regular constituent of agglutinin precipitates, but was found constantly in precipitates of lipolysin.

8. This association with lipolysin makes possible the localization of both copper and ferment in the cortex of the egg.

9. The association of copper with lipolysin is not an isolated case. Copper was found also in preparations of pancreatic enzymes and pepsin.

10. In eggs exposed to a $n/180$ Cu solution for twenty minutes the copper is widely diffused through the cytoplasm and concentrated in the chorion, the vitelline membrane, and the cortex.

11. Approximately the amounts of copper normally present in 1 c.c. of *Arbacia* eggs are as follows:

Unripe ovarian eggs	= 17 μgr.
Ripe shed eggs	= 175 μgr.
Fertilized eggs	= 21 μgr.

12. From the preceding and other considerations it is suggested that copper, in general, may be more than an adventitious element, physiological only in pigments, and merely tolerated in all other connections.

13. The association of copper with enzymes is explained as the outcome of some sort of union, very likely chemical, between enzymes or co-enzymes, or both, and the metal. It is also suggested that possibly the differences in the proportionality between silver and mercury effects, as well as the Danysz recovery after "poisoning," may be due to the distribution and subsequent redistribution of the metallic ions between enzymes and co-enzymes.

14. The oligodynamic action of copper is explained as due to the inactivation of that fraction of the enzyme or co-enzyme which was not normally inactivated by the copper present in the first place. If inactivation is produced by silver, it is suggested that the total inactive enzyme or co-enzyme would be composed of two fractions—the one inactive because of the normal copper content, the other because of the silver added.

15. It is suggested that the concentration of copper in the ovum at maturity may have something to do with limiting the growth of the egg; that the elimination of copper-bearing pigment during

fertilization may indirectly restore or produce conditions essential for further growth and development.

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CONTENTS

Smithsonian Institution
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ZELENY, CHARLES. *The Temperature Coefficient of a Heterozygote with an Expression for the Value of a Germinal Difference in Terms of an Environmental One* . . . 105

SIVICKIS, P. B. *Studies on the Physiology of Reconstitution in Planaria lata, with a Description of the Species* . . . 113

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BIOLOGICAL BULLETIN

THE TEMPERATURE COEFFICIENT OF A HETEROZYGOTE WITH AN EXPRESSION FOR THE VALUE OF A GERMINAL DIFFERENCE IN TERMS OF AN ENVIRONMENTAL ONE.[1]

CHARLES ZELENY.

It has been shown by Seyster and Krafka that the size of the eye and the number of its ommatidia in "bar-eye" *Drosophila* varies with the temperature at which the larvæ are developed. An increase of one degree Centigrade produces on the average a decrease of about 10 per cent. in ommatidial number. In ultra-bar, an allelomorph of bar and full, the change is about 8 per cent. per degree. On the other hand, full eye has a much lower temperature coefficient. Counts being made at present by Miss Karrer show a change of only 2½ per cent. per degree.

Since the effect upon bar and ultra-bar is so much different from that upon full, it becomes a matter of interest to determine the reaction of the heterozygotes. Are they intermediate in this respect as well as in ommatidial number? The results may be expected to throw some light upon the manner of reaction of the genes and on the nature of dominance.

The present report deals with the ultra-bar heterozygotes.

The effect of temperature upon ultra-bar homozygotes has been determined by Krafka (1920, p. 416). His values are copied in the next to the lowest line of Table I. From these values the average effect of one degree of change in temperature may be determined as follows: The average ommatidial number is 51.5 at 15° and 15.8 at 30°. The difference between the logarithms of 51.5 and 15.8 divided by fifteen and reduced to its arithmetical value is 1.080. In other words, one degree decrease in temperature between 30° and 15° causes an average increase of 8 per cent. in ommatidial number. Between 27° and 15° the average increase is 7.6 per cent.

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[1] Contribution from the Zoölogical Laboratory of the University of Illinois. No. 210.

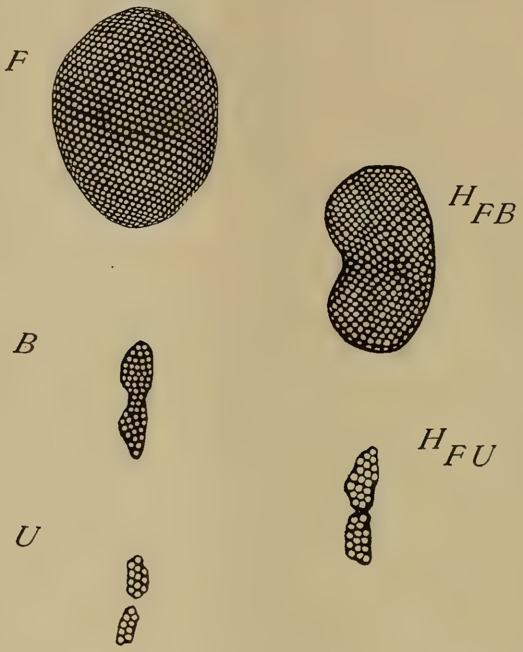

FIG. 1. *F, full eye. B,* bar. *U,* ultra-bar. H_{FB}, heterozygote of full and bar. H_{FU}, heterozygote of full and ultra-bar.

In some work not yet published, but which she has allowed me to use for the present purpose, Miss Karrer finds that full eye has an average increase of only about 2½ per cent. per degree between 29° and 15°.

TABLE I.

	Number of Ommatidia.			
	15.	20.	27.	30.
Two full eye factors. Homozygous full eye	1,077.5	947.0	810.6	754.9
One full eye and one ultra-bar factor. Heterozygote	112.1	72.1	40.5	37.1
Two ultra-bar factors. Homozygous ultra-bar	51.5	32.6	21.3	15.8
Difference between heterozygote and homozygous ultra-bar	60.6	39.5	19.2	21.3

Heterozygotes Exhibit a Complete Change in Reaction from the Full to the Ultra-bar Type.—In the case of heterozygous females, containing one full and one ultra-bar factor, the mean ommatidial values as determined by the present experiments are given in the second line of Table I. There are 37.1 ommatidia at 30°, 40.5 at 27°, and 112.1 at 15°. The average increase per degree determined by the method previously described is 7.7 per cent. between 30° and 15° and 8.9 per cent. between 27° and 15°. These values are strikingly different from 2.5 per cent., the value for full eye, and strikingly similar to 8.0 and 7.6, the values for homozygous ultra-bar.

The temperature coefficient of the heterozygotes must, therefore, be considered as essentially like that of the ultra-bar parent and wholly different from that of the full parent. While a single ultra-bar factor is not sufficient to bring about the complete effect in reducing ommatidial number as produced by two ultra-bar factors, it is sufficient to produce the complete change to a physiological system of the ultra-bar type.

The change in ommatidial number with change in temperature can be explained most satisfactorily by assuming a differential effect of temperature upon the physiological processes involved in ommatidial production as opposed to other physiological processes. In view of the fact that temperature is effective only during a few hours of larval life, it may be considered that the initial steps in the formation of ommatidia are confined to a definite embryological period. The length of this period is determined by the general physiological processes of the larva, while the rate of formation of ommatidia during the period is a function of special processes which have a different coefficient. It is evident that under these circumstances two different temperatures must give two different ommatidial numbers. The difference in the temperature coefficients is slight in full eye and the mutation to bar or ultra-bar involves a marked increase of this difference. Further analysis awaits a more accurate knowledge of the nature of the embryological processes involved. Whatever the character of these processes, however, it is clear that the reaction system produced by the introduction of a single ultra-bar factor is of the ultra-bar type, even though the reduction in ommatidial count at any specific temperature is not as great as that produced by two ultra-bar factors.

The analysis makes it evident that there are two distinct processes involved in the mutation from full to ultra-bar. One of these consists in an essential change in the type of reaction as shown by the change in the temperature coefficient. The other process involves a change in the general level of the rate of the reaction without affecting its specific character. The first is fully accomplished by a single ultra-bar factor. The second is influenced quantitatively by the number of ultra-bar factors.

The Effect of Temperature upon Dominance.—Since the heterozygote has the same temperature coefficient as homozygous ultra-bar, and one that is much greater than that of homozygous full eye, it becomes a matter of interest to consider the effect of temperature upon dominance. Elsewhere (1920, p. 308) I have discussed a method of determination of the coefficient of dominance by the use of a factorial scale in which the effect of a degree of temperature is taken as the measure of an unit factor. It is obvious that on this basis there can be no change in dominance with temperature, because the ommatidial value of the unit varies with change in effect of temperature.

If, however, it is desired to get an expression for dominance which is based directly upon the somatic expression—*i.e.*, upon the ommatidial number—such a value changes with the temperature. Suppose that complete or 100 per cent. dominance of full is a condition in which the heterozygote has the same ommatidial number as full eye and complete recessiveness or zero per cent. dominance of full a condition in which the heterozygote has the same ommatidial number as ultra-bar. Likewise suppose that the ommatidial count is the scale of values. Then the coefficient of dominance of full as expressed on a percentage basis is

$$\text{C.D. } F = \frac{H - B'u}{F - B'u} \times 100,$$

in which H is the ommatidial count of the heterozygote, $B'u$ that of ultra-bar, and F that of full. Correspondingly

$$\text{C.D. } B'u = \frac{F - H}{F - B'u} \times 100.$$

In this way the coefficient of dominance of full eye is readily determined as 5.9 per cent. at 15°, 4.3 per cent. at 20°, 2.4 per cent. at 27°, and 2.9 per cent. at 30°. The general decrease with increase in temperature is evident. The reversal between 27 and 30 may probably be explained by the disturbance resulting from an approach to the maximum temperature.

The Value of a Germinal Factor in Terms of an Environmental One.—Perhaps the most interesting point in connection with the present data is the demonstration that they furnish of the fact that the gene, ultra-bar, has the same type of reaction as a temperature difference. It is possible to state the effectiveness of particular germinal factors in terms of the corresponding effects of temperature. Such an attempt has been made in previous studies of the bar races and the temperature coefficient has given the basis for the evaluation. Since these previous studies had shown that a change of one degree in temperature produces a change of approximately 10 per cent. in ommatidial number, a "unit" factor was, for convenience, taken as one that produces the same change. The units in the factorial scale, whether environmental or germinal, are thus expressed on the same basis.

First of all, it will be well to take up the demonstration of the fact that the particular germinal difference represented by the addition of a second ultra-bar factor in place of the full factor of the heterozygote does not correspond to a constant somatic expression. The difference between heterozygous and homozygous ultra-bar is not represented by a constant difference in ommatidial number. Thus at 15 degrees the difference is 60.6 ommatidia, at 20 degrees 39.5, at 27 degrees 19.2, and at 30 degrees 21.3. These data are given in Table I. and in graphic form in Fig. 2, where the lengths of the heavy vertical lines are proportional to the ommatidial differences at the various temperatures. The marked change with temperature is obvious, though the germinal difference remains constant. The ommatidial difference can not, therefore, serve directly as a measure of germinal difference.

If, however, the unit of measurement is the effect produced by a degree of change in temperature, 8 per cent. in this case, and the effect produced by the substitution of the second ultra-bar factor for the full factor of the heterozygote is measured in terms of this unit, the result obtained is shown in Table II. and graphically in Fig. 3. In the latter the scale at the left represents the logarithms

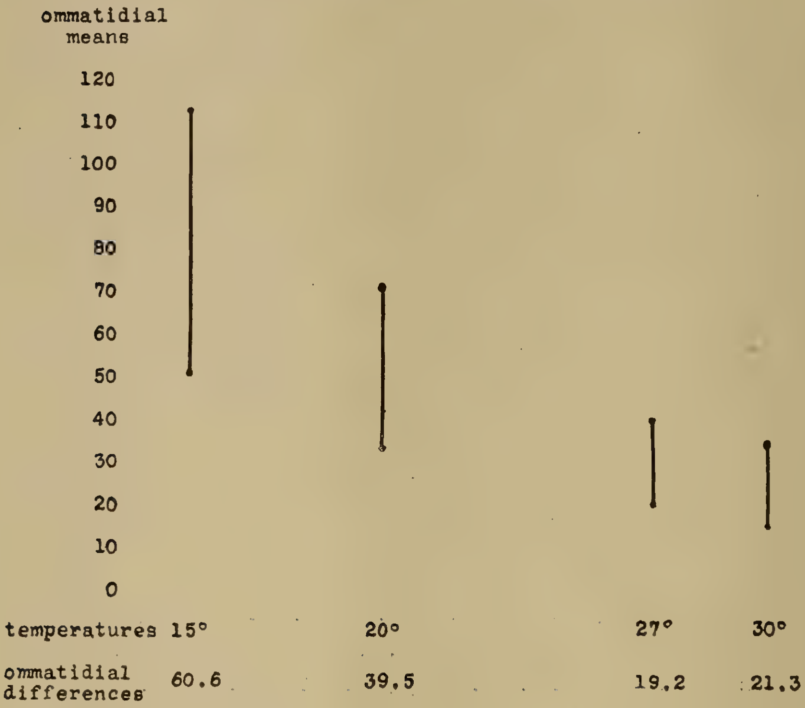

FIG. 2. The length of each heavy vertical line represents the difference in ommatidial number between homozygous and heterozygous ultra-bar at the respective temperature. The numerical values are given at the bottom of the figure. The heterozygotes have one full and one ultra-bar factor. The scale at the left represents ommatidial numbers. The marked change in the ommatidial difference with change in temperature is to be noted.

TABLE II.

FACTORIAL VALUES.

	Eight Per Cent Units.			
	15°	20°	27°	30°
One full eye and one ultra-bar factor. Heterozygote	+25.3	+19.6	+12.3	+11.0
Two ultra-bar factors. Homozygous ultra-bar	+15.3	+ 9.4	+ 3.9	0.0
Difference in eight per cent. units or temperature units	10.3	10.2	8.4	11.0

of the ommatidial counts, arranged so that each unit has the same logarithmic value, corresponding to a change of 8 per cent. in ommatidial number. The location of the zero point is of course purely arbitrary.

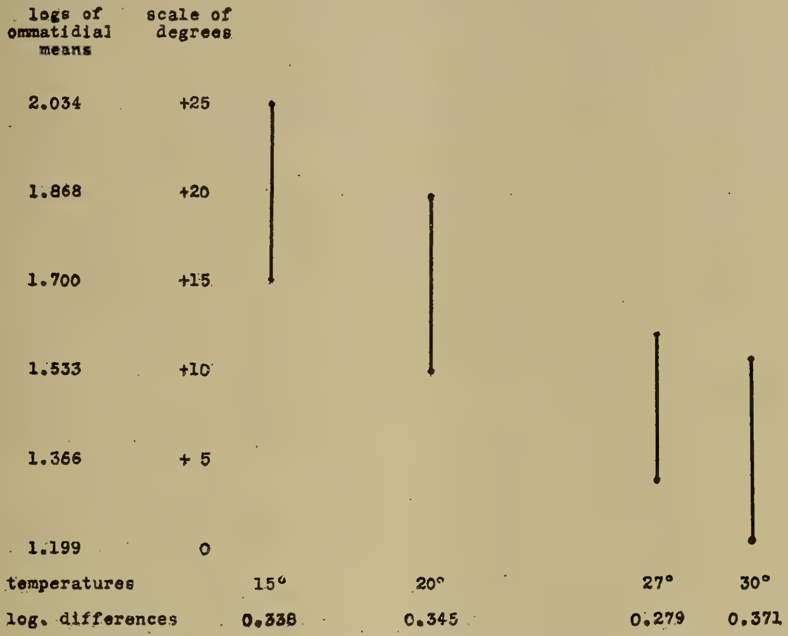

FIG. 3. The length of each heavy vertical line represents the difference in terms of logarithms of ommatidial number between homozygous and heterozygous ultra-bar at the respective temperatures. The numerical values are given at the bottom of the figure. The heterozygotes have one full and one ultra-bar factor. The logarithmic scale is represented at the left. In the next column is the scale of corresponding degrees Centigrade which would produce the same effect, starting with an arbitrary zero at the lowest value of the present observations. It is to be noted that the difference between homozygote and heterozygote on this scale is fairly constant.

The heavy vertical lines again give the effect produced by the substitution of a second ultra-bar factor for the full factor of the heterozygote at the different temperatures. The second ultra-bar factor at 15° C. depresses the value by an amount equal to that produced by 10.3 degrees of temperature, at 20° C. by 10.2 degrees, at 27° C. by 8.4 degrees, and at 30° C. by 11.0 degrees. The average depression is the same as that produced by ten degrees of increase in temperature, and considering the character of the determinations the values are remarkably constant. The conclusion may be safely made, therefore, that a proper measure has been found for the expression of the germinal value in question. A homozygous ultra-bar at 15° has the same ommatidial number as a heterozygote at 25° and a homozygote at 20° the same number as a heterozygote at 30°.

On the logarithmic scale of ommatidial number it is, therefore, possible to express the relation between the germinal factor and the environmental factor as a constant. The present data, therefore, strengthen the validity of the use of this scale as a measure of the germinal factors as well as the environmental ones.

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STUDIES ON THE PHYSIOLOGY OF RECONSTITUTION IN *PLANARIA LATA,* WITH A DESCRIPTION OF THE SPECIES.

P. B. SIVICKIS,

Hull Zoölogical Laboratory, University of Chicago.

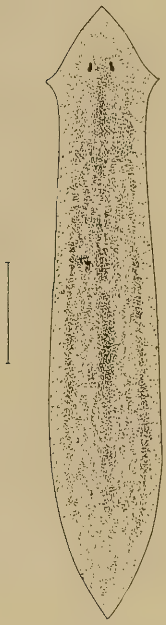
The earlier work on the reconstitution of isolated pieces of planarians into new individuals was very largely concerned with the description of the visible changes, such as the outgrowth of new tissue, its differentiation, the reorganization and redifferentiation of the old parts, the changes in shape, the minimum size of pieces capable of reconstitution, and the relations between the polarity of the new individual and that of the animal from which the piece was taken. Most of the experiments were performed with small numbers of pieces, often from different regions of the body and without any attempt at a physiological standardization of the experimental material. During the past twenty years *Planaria dorotocephala* has been extensively used in this laboratory as material for experimental investigation, and in the course of this work various methods have been developed which have made possible some degree of physiological analysis of the process of reconstitution in this species. In this work physiological standardization of material, mass experiments, and control of environmental conditions have played an important part.

The desirability of including other species within the program of investigation has become increasingly evident with the progress of the work on *P. dorotocephala,* and at the suggestion of Dr. C. M. Child the analysis of reconstitution in a closely related species was begun. The present paper comprises a part of the results of this investigation.

In this connection I take the opportunity to acknowledge my deep indebtedness to Professor Child for his advice, friendly criticism, and revision of the manuscript. I also wish to express my thanks to Dr. L. H. Hyman for the data on oxygen consumption presented in this paper and for many helpful suggestions.

MATERIAL: DESCRIPTION OF SPECIES.

In many rivers and lakes about Chicago a planarian, commonly identified in the past as *P. maculata,* occurs in large numbers. Even a cursory examination makes it evident that this form differs in various respects from *P. maculata* of the Atlantic slope. Attention has already been called to these differences by Hyman ('20). A comparison of living individuals of this form and *P. maculata* from the region of Woods Hole, Mass., shows the following differences: The pigment pattern of the mid-western form (Fig. 1) is distinctly coarser and more irregular; the individual pigment spots and the unpigmented areas are more clearly visible to the naked eye than in *P. maculata* (Fig. 2). In a mixed stock the two



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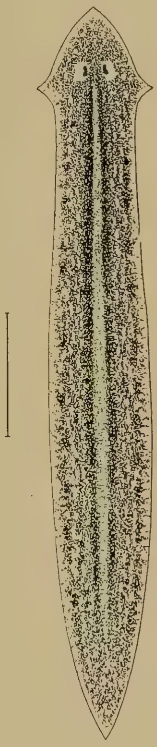

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FIG. 1. *Planaria lata* n. sp.

FIG. 2. *Planaria maculata.*

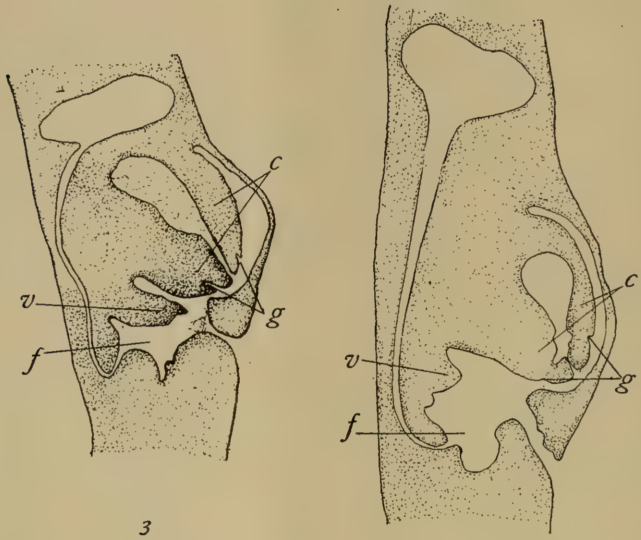
forms are at once distinguishable by these differences in pigmentation. Moreover, the general color effect to the naked eye in the mid-western form is a light grayish brown, mottled or dappled, while in *P. maculata* the brown tint is much deeper and more uniform. In *P. maculata* a light median longitudinal stripe is almost invariably present (Fig. 2), while the mid-western form usually shows an obscure dark median stripe (Fig. 1).

As regards shape of body, the mid-western form is distinctly broader in proportion to length than *P. maculata* (cf. Figs. 1 and 2). This difference is evident during locomotion as well as at rest. The cephalic lobes are apparently slightly less developed and the digestive tract appears more highly branched in the mid-western form than in *P. maculata.*

As regards motor behavior, also marked differences appear. The mid-western form is distinctly more sluggish, reacts more slowly, and progresses less rapidly than *P. maculata.* Similar differences in reaction to food exist. *P. maculata* can be collected by placing pieces of meat in the water in localities where the species occurs. As the extractives diffuse, the animals will come to the meat from a distance of several inches in standing water and from greater distances in flowing water. The mid-western form can not be collected in any considerable numbers in this way because only those animals immediately about the meat react. In feeding stocks in the laboratory it has been found necessary to grind the meat and spread it over the bottom of the container, instead of placing pieces at intervals of two or three inches, as has been the practice with *P. dorotocephala* and *P. maculata.*

All these differences persist unchanged in stocks of the two forms kept in the same water and under the same conditions in Chicago. This is true, not only as regards the original individuals, but also as regards young animals resulting from fission or reconstitution of pieces and animals hatched from eggs. Reconstitution experiments with the two forms also show certain characteristic differences in head frequencies.

These differences are unquestionably sufficient to make it evident that the mid-western form is not *P. maculata.* However, since the morphological characteristics of the organ complex of the genital atrium, and particularly the copulatory organ, are commonly regarded as the most trustworthy criterion of specific differences, sections of this region of sexually mature animals have been made.



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Figs. 3, 4. Atrial genital complex of the two species of *Planaria:* Fig. 3, *P. lata;* Fig. 4, *P. maculata* combined from two adjoining sections; *c,* copulatory organ; *f,* female region of atrium; *g,* circular groove or furrow on copulatory organ; *m,* male region of atrium; *v,* valve or fold between the two parts of atrium.

Fig. 3 is a median sagittal section through this region of the mid-western form and Fig. 4 a similar section from *P. maculata,* both from sexually mature animals. The circular groove (*g*) about the apical region of the copulatory organ (*c*) is deeper in *P. lata* and the outline of the fold or valve (*v*) between the common portion of the atrium and the female duct (*f*) is very different in the two species. Other differences in shape of the parts and cavities are evident, but may be due in part to differences in muscular contraction. The differences in the atrial complex confirm the conclusion that the two forms are different species, and since the mid-western form does not agree with descriptions of other species already given, it is evidently an unnamed species and is named and described as follows:

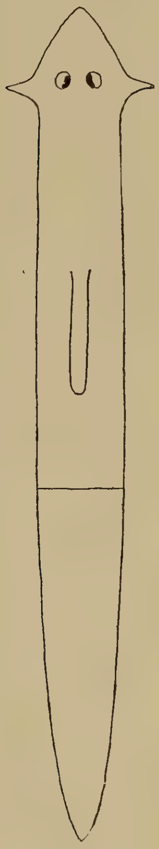
Planaria lata n. sp.—Length of full-grown, sexually mature individuals averaging 12–14 mm., occasionally 16 mm. Body relatively broad, in full-grown animals width from tip to tip of cephalic lobes and from margin to margin at mouth about one sixth of length. Pigment pattern coarse, irregular, often with obscure dark median stripe, the general effect being mottled or dappled light grayish brown. Cephalic lobes short. Animal sluggish and markedly less sensitive to external factors than either *P. dorotocephala* or *P. maculata.* Structure of organ complex of genital atrium as in Fig. 3.

The present paper is primarily concerned with this species, but attention is called to the physiological differences between this species and *P. dorotocepha* which have been brought to light by the experiments.

The Occurrence of Fission and Sexual Maturity.

Like *P. dorotocephala, P. lata* shows no visible morphological indication of the presence of a posterior zoöid, but, as will appear below, the presence of such a zoöid can be demonstrated by physiological methods. Fission in *P. maculata* has been described by Curtis ('02) and the act of fission has been observed in *P. dorotocephala* by Child ('10, '11*c*). In the latter species it consists in an independent motor reaction of the posterior zoöid while the animal is moving forward. The posterior zoöid attaches itself, the anterior zoöid continues to advance, and the body in front of the attached portion is finally ruptured. Fission is much more likely to occur after slight stimulation than after violent disturbance, for in the latter case the posterior zoöid is controlled by the anterior and does not react independently. The act of fission has not been observed in *P. lata,* but undoubtedly it occurs in the same manner. In the laboratory fission is often induced by changing the water, but does not occur at once, while the animals are very active, but only later as their activity decreases.

As regards the level of the body at which fission occurs, *P. lata* differs markedly from *P. dorotocephala.* In the latter species fission normally occurs at a level 1–3 mm. posterior to the mouth (Fig. 5), and in cases of delayed fission in the laboratory the posterior fission piece may be longer than the anterior. In *P. lata,* however, fission takes place much nearer the posterior end (Fig. 6), and in animals 12–13 mm. in length the posterior fission piece is only 2–3 mm. in length, and in shorter animals it is not only absolutely, but relatively, shorter. In *P. dorotocephala* the posterior fission piece often divides again after four or five days, when it begins to move about more or less normally. Apparently at this stage the developing head is unable to control the whole length of the posterior fission piece and fission takes place at one of the more posterior zoöid boundaries. Such second fission has never been



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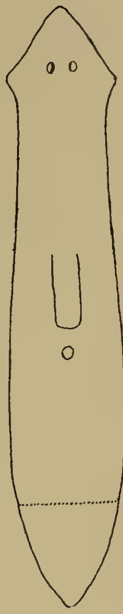

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FIG. 5. *P. dorotocephala,* showing level at which fission usually occurs.

FIG. 6. *P. lata,* showing level at which fission usually occurs.

observed in *P. lata,* but the susceptibility (p. 32) and the head frequency (p. 37) of the region just anterior to the level of fission suggest that some slight degree of physiological isolation and the earliest stages of another zoöid exist there in large animals. If such a zoöid is present, it develops to a stage at which fission is possible only after fission has occurred posterior to it and it has become the posterior end of the body.

The maximum length attained by *P. lata* under ordinary conditions in nature and in the laboratory is twelve to fourteen millimeters, and at this size the animals become sexually mature, even though fission continues to occur. The level of fission is so far posterior to the genital pore that the development of the ducts and pore is usually not affected to any appreciable degree by fission. In this respect also this form differs from *P. dorotocephala,* in which the level of fission is so near the level of the genital pore that the occurrence of fission in an animal approaching sexual maturity usually brings about disappearance of the pore and at least the posterior portions of the ducts. In the localities about Chicago *P. lata* becomes sexually mature and deposits eggs from June to September. In the laboratory maturity and egg laying may occur at any time of year. In *P. dorotocephala* sexual maturity may occur in the laboratory when the animals are well fed and fission is prevented by keeping them on slimy or vaselined surfaces, but it has not been observed under natural conditions in this region, being apparently prevented by fission and perhaps also by periodic starvation (Child, '11*c*). Curtis also found that in some localities *P. maculata* does not become sexually mature, but did not discover the determining conditions. It may be suggested that an environment which inhibits slightly the physiological activity of the animals and so decreases the range of dominance may result in the occurrence of fission at a more anterior level, and this may interfere with the development of the genital ducts and pore. Experiments to test this suggestion have not yet been performed.

METHODS.

The animals are found both on stones at the bottom and on *Elodea* and other water plants at various levels. On the stones all sizes from very young to sexually mature animals and numerous egg capsules may be found in summer, but among the plants only the smaller, younger animals have been collected. Stocks are kept in the laboratory for three or four weeks before using experimentally so that uniformity in nutritive conditions may be approximated. They are fed three times a week with ground and washed beef liver, as described by Hyman ('20).

The work in this laboratory with planarians has demonstrated that in a stock of animals collected at one time, kept under as nearly as possible identical conditions of temperature, nutrition, water supply, etc., size is the best criterion of physiological condition, and particularly of physiological age, as indicated by susceptibility and respiratory rate, which can readily be applied to the living animals in the selection of material for experiment. Uninjured animals of the same size from such a stock show a high degree of uniformity in susceptibility to chemical and physical agents and in rate of respiration, as shown by the work of Child, Hyman, Behre, and Buchanan, and are more alike physiologically than material selected on any other basis thus far discovered. In these animals the amount of growth, whether rapid or slow, and consequently the size of the individual, is a far more exact measure of their physiological age, and so of their susceptibility and rate of respiration, than the length of time they have lived as individuals (Child, '15*a,* Chap. IV.; Hyman, '19 *C*).

Since the experiments recorded in this paper are all mass experiments—*i.e.,* with numbers of individuals—the material for each experiment is selected on the basis of size. Such standardization of material is necessary for the attainment of definite results which can be predicted and controlled and it makes possible prediction and control to a high degree. In the course of the work experiments were performed with standardized material from general stocks collected and kept as described above, from stocks composed of animals hatched from eggs in the laboratory, and from stocks grown from cut pieces.

The experimental data presented below concern chiefly respiration, susceptibility, and head frequencies—*i.e.,* the frequencies of occurrence of the various forms of head in the reconstitution of pieces in relation to level of body, length of piece, and physiological age of animals. Some experiments on modification and control of head frequency by means of chemical agents have been performed, but are only briefly mentioned.

The data on respiration include comparative estimations of CO_2 production by colorimetric determination of pH with phenol-sulphone-phthalein as indicator and the indicator-buffer solutions made up by Hynson Westcott and Dunning as standards. In these experiments lots of as nearly as possible equal weights of animals or pieces to be compared are sealed in pyrex tubes of the same diameter as the standard tubes in equal volumes of indicator solution of the same concentration as the standard tubes and the change in color recorded at regular intervals and usually also to a definite pH. Some data on oxygen consumption determined by the Winkler method are also given. For these I am indebted to the kindness of Dr. Hyman.

In the experiments on susceptibility KNC has been chiefly used as agent, since it has been abundantly demonstrated that with proper precautions susceptibility to KNC can be used as a general comparative measure of physiological condition and particularly of rate of respiration in *Planaria*.[1] In the present study the susceptibility method has been used for two purposes: first, for demonstration of the axial gradients and the posterior zoöid by the differential susceptibility of different levels of the body; second, to demonstrate the changes in physiological condition of pieces following section. The general susceptibility gradients are shown in figures and the comparative susceptibilities of pieces by graphs. The method of graphing the data is described in connection with the data.

In the experiments on reconstitution the body posterior to the head is cut into a certain number of pieces—four, six, eight, sixteen—according to the experiment. Animals of the same size are used for each experiment and the pieces from each individual are cut as nearly as possible the same length, the more extreme irregularities being discarded. Consequently the corresponding pieces from different individuals represent as nearly as possible the same region of the body. All corresponding pieces—*i.e.*, those representing the same body region—are kept together in one container. The usual number of pieces in each lot is fifty, each taken, of course, from a different individual of the size used in that experiment. The pieces are allowed to undergo reconstitution and examination is made and results recorded after twelve to fourteen days, at which time reconstitution is so far advanced that no further change in the character of the form produced will occur. Since the forms produced fall into certain groups or types, as described in a following section, the results can be tabulated to show the frequency of each type in each lot. In this species, as in *P. dorotocephala,* the head is the most characteristic distinguishing feature of the different forms produced, except in very short pieces. The basis of tabulation is, therefore, form and structure of the anterior end, and the tables show the frequencies of the different forms and are called, for convenience, head frequency tables, this term having been used for similar data on *P. dorotocephala.* From the tabulated head frequencies graphs are constructed by a method described below, and in this way the head frequencies in different regions, in pieces of different lengths, and in animals of different size or physiological condition may be directly compared.

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[1] Child, '14*a, c,* '15*a,* chaps. III.–VII., '16, '19*a, b;* Hyman, '19*a, b, c,* '20, '22.

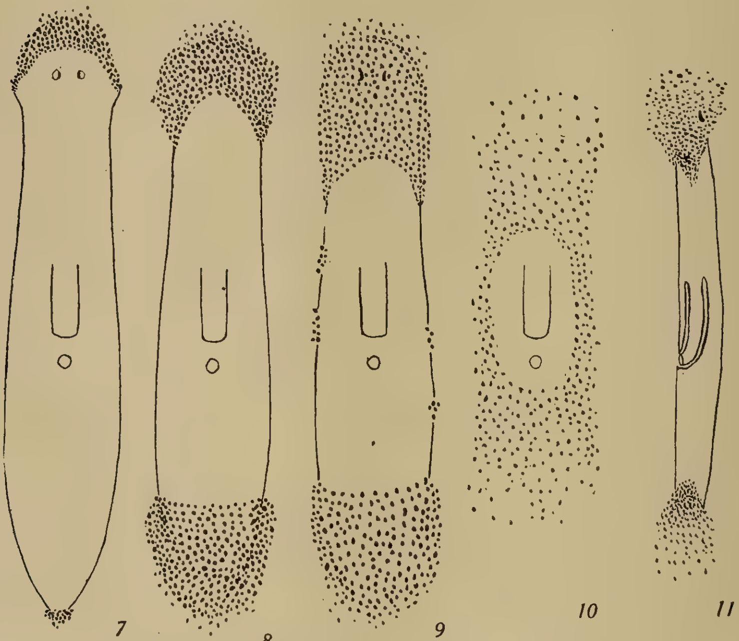
The relation of head frequency to region of body and length of piece is shown by comparing pieces of different lengths and from different body levels of animals of the same size, and therefore approximately of the same physiological age. The relation of head frequency to physiological age is determined by comparing the results obtained with pieces from animals of different lengths. In my experiments animals of two standard lengths have been chiefly used: full-grown animals, 11–13 mm. long, physiologically old, and with low rate of respiration; and young growing animals, 4–6 mm. long, with much higher rate of respiration. The smaller size is the smallest which can be conveniently used for such experiments because of the difficulty of cutting pieces of equal size in the smaller individuals. Some experiments have been performed with sizes intermediate between these two extremes, some with animals raised from eggs and some with animals raised from cut pieces.

The Axial Susceptibility Gradients.

The occurrence of definite axial susceptibility gradients in both animals and plants and their relation to metabolic rate has been discussed in various publications (*e.g.*, Child, '20*b*). Various lines of evidence show that susceptibility to a wide range of chemical agents in concentrations or intensities above the limit of tolerance or acclimation and below the limit at which death occurs immediately varies in general directly with metabolic rate, or more specifically with rate of respiration. Susceptibility gradients are characteristic features of physiological axes in both plants and animals and many facts indicate that such axes are primarily quantitative physiological gradients.

It has been found that in *P. dorotocephala* the head frequencies at different levels of the body show a definite relation to the polar susceptibility gradient, regions of high susceptibility being regions of high head frequency and vice versa. The observations on susceptibility in *P. lata* show that a similar relation exists in this species. In these observations KNC was used in most cases as agent, because it is known to be a powerful inhibitor of oxidations and has been extensively employed in the study of susceptibility, but many other agents of very different constitution—*e.g.*, $HgCl_2$, $CuSo_4$, acetic acid, various anesthetics, etc.—in proper concentration give essentially the same results. The procedure consists, first, in determining a concentration which kills slowly enough to permit the differences in susceptibility to appear clearly and which is not low enough to permit acclimation, and, second, in observing the progress of disintegration of the tissues of animals placed in the solution, in closed containers if necessary to avoid loss from volatilization. As death of any part occurs or approaches structural disintegration of the tissues takes place, and such disintegration appears first in certain regions and follows a definite course, so that certain body regions are completely disintegrated while others are still intact and moving.

The general course of disintegration in *P. lata* is shown in Figs. 7–10. The head and the posterior zoöid are most susceptible and disintegration progresses posteriorly from the head and at the same time involves a region anterior to the posterior zoöid and posterior to the genital pore, the last region to disintegrate being that between the mouth and the genital pore. Commonly the lateral margins of the body disintegrate somewhat earlier than median regions at the same level, but in some individuals these transverse differences do not appear, and in some the median region apparently disintegrates earlier than the margins. There is not much difference between dorsal and ventral, but ventral regions usually disintegrate slightly in advance of dorsal (Fig. 11).



FIGS. 7–11. Susceptibility gradients in *P. lata,* as shown by KNC *m*/1000: Figs. 7–10, stages in progress of disintegration in dorsal view; Fig. 11, a stage of disintegration in lateral view to show difference between dorsal and ventral.

It should be noted that these statements concern primarily the body wall, but they appear to hold good for the parenchyma also. In well-fed animals the digestive tract is highly susceptible and, even though the cyanide must pass through the body wall to reach it, the digestive tract usually swells and disintegrates earlier than the body wall and often bursts through the latter at various points. In starved animals the digestive tract is much less susceptible and in advanced starvation may remain intact longer than the body wall.

In general, the course of disintegration in this species is very similar to that in *P. dorotocephala,* but some minor differences appear. In long individuals of the latter, possessing more than one zoöid in the posterior region, the different zoöids usually show different susceptibilities (Child, '11*c*, '13*b*). The early disintegration of the region anterior to the posterior zoöid in *P. lata* may indicate the presence here of another zoöid at a very early stage; in other words, some degree of physiological isolation with increase of metabolic activity may exist anterior to the level of fission and yet be insufficient to permit fission at this level.

In *P. dorotocephala* the margins of the body always disintegrate in KNC before median regions, while in *P. lata* this may or may not be the case. These differences are apparently associated with the specialization of the margins as motor organs, and particularly as organs of secretion of slime. There are many glands in this region and the alkaline cyanide stimulates these to increased activity. Often the separate glands disintegrate before other parts of the margins, particularly in *P. dorotocephala.* Apparently the margins of *P. lata* are less highly specialized in this way, for the glands appear less distinctly as regions of disintegration and there is less motor activity of the margins. In neutral or acid cyanide and in other acid agents the glandular activity is apparently not increased and motor activity is decreased, and in such solutions the susceptibility of the margins, even in *P. dorotocephala,* is usually less than that of median regions.

In *P. dorotocephala* the dorsal body wall disintegrates earlier than the ventral in alkaline cyanide. The dorsal wall is thinner than the ventral and shows many localized regions of disintegration, apparently glandular. In acid agents ventral regions are usually more susceptible. In *P. lata* the specialization of the dorsal surface is apparently less, so that even in alkaline agents the difference between dorsal and ventral is slight and the ventral surface is usually the more susceptible.

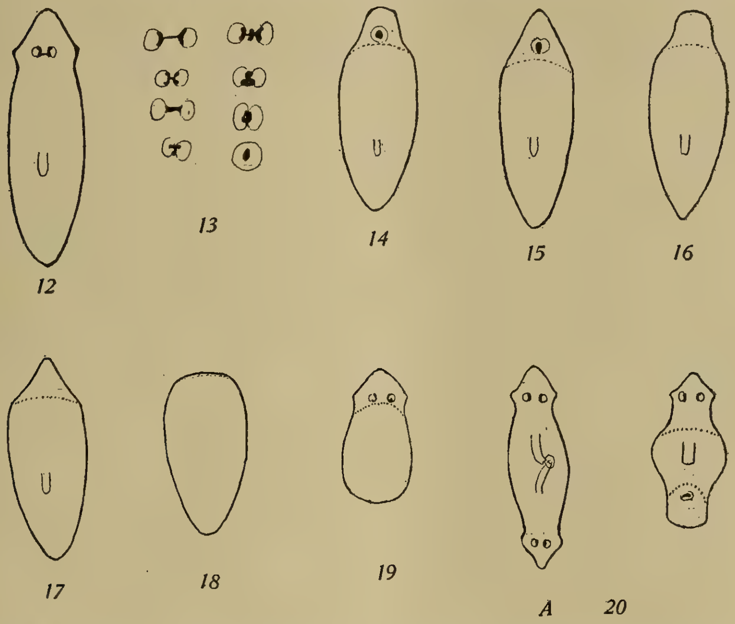
In the early developmental stages of turbellaria the median ventral region is more susceptible than lateral and dorsal regions (Child, unpublished), and in full-grown planarians the new tissue develops more rapidly from the median ventral region than from other parts of a cut surface. This fact suggests that internally the median ventral region still possesses the highest metabolic rate. Apparently the primary symmetry gradients undergo more or less alteration in the body wall of some species, and the susceptibility data indicate that such alteration is greater in *P. dorotocephala* than in *P. lata.* The latter species seems to retain more nearly the characteristics of earlier stages.

Young animals are always more susceptible than old and the differences in susceptibility at different levels of the body are less in the young. In fed animals susceptibility decreases from the time of hatching to maturity and in this respect parallels rate of respiration (Child, '19*a*; Hyman, '19*c*). In full-grown animals the time from the beginning to the end of disintegration in *m*/1,000 KNC at 20° C. is eight to ten hours; in young animals it is much less, increasing with advancing age. High temperature increases, low temperature decreases susceptibility. Starvation increases susceptibility and also decreases the differences at different levels. In all these respects the two species are alike.

The Forms Resulting from Reconstitution.

As in *P. dorotocephala,* the results of reconstitution differ in definite and orderly ways according to length of piece and region of body from which it is taken. Some of these differences, such as the level at which the new pharynx appears, length of prepharyngeal and postpharyngeal regions, are merely temporary features of the earlier stages of reconstitution, and are later largely or completely obliterated by differential growth of prepharyngeal and postpharyngeal regions, particularly if the new individuals are fed, until finally all normal or nearly normal individuals attain approximately the same proportions.

The most conspicuous differences in the results of reconstitution concern the head and these differences are, with certain rare exceptions, permanent. Isolated pieces do not always develop anterior ends like that of the normal animal in nature, but abnormal forms occur which constitute a continuous series with some secondary modifications from the normal head to a completely headless condition. The members of this series fall readily into the same types or groups as in the case of *P. dorotocephala* (Child, '11*a*, '21), viz., normal, teratophthalmic, teratomorphic, anophthalmic, acephalic. The normal head is like the head of Fig. 1. The teratophthalmic head is normal in outline, but the eyes are more or less approximated to the median line and the pigment spots are often partially united or even fused (Figs. 12, 13). In the teratomorphic head there is a single, or apparently single, median eye and the anterior region between the cephalic lobes is incompletely developed, so that the lobes appear more or less anteriorly or fused at the median line (Figs. 14, 15). The anophthalmic head is merely an outgrowth of tissue without eyes (Figs. 16, 17), and in the acephalic form the anterior end simply heals over without outgrowth (Fig. 18). As regards degree of development, the cephalic ganglia of these forms also constitute a continuous series from normal ganglia in the normal head to a rudimentary ganglion in



FIGS. 12–20. Forms resulting from reconstitution: Figs. 12, 13, teratophthalmic heads and eyes: Figs. 14, 15, teratomorphic heads; Figs. 16, 17, anophthalmic heads; Fig. 18, acephalic form; Fig. 19, tailless form; Fig. 20, biaxial heads.

the anophthalmic, and no ganglia in the acephalic form (Child and McKie, '11). In this species, as in *P. dorotocephala,* no other head forms have been observed except secondary modifications of some one of these forms in acclimation to, or recovery from, inhibiting agents (Child, '21) and inequalities or asymmetries in position of eyes resulting from oblique section or other incidental conditions and usually temporary. The frequency of occurrence of these various forms in any lot of pieces is the head frequency of the lot, and the following experiments on reconstitution are chiefly concerned with the relations of head frequency to length of piece, level of body, and size of animal from which pieces are taken.

The results of reconstitution in very short pieces require mention. Forms with heads, usually normal, but without posterior ends, "tailless forms" (Fig. 19) and "biaxial heads" (Fig. 20), appear rarely in sixths of large animals, more frequently in eighths, and their frequency increases with decreasing length of piece to the limit of length at which wound closure and reconstitution fail to occur. Under ordinary conditions these forms are much more frequent and appear in longer fractions of the body in this species than in *P. dorotocephala.* In the latter species they have never been seen in sixths or eighths, except rarely in eighths from small young animals, and they are rare even in sixteenths and twentieths of large animals.

HEAD FREQUENCIES IN RELATION TO LENGTH OF PIECE, LEVEL OF BODY, AND PHYSIOLOGICAL AGE OF ANIMAL.

The data presented in this section include head frequencies of fourths, sixths, eighths, and sixteenths of full-grown animals 11–13 mm. in length and fourths and sixths of young animals 4–6 mm. in length. In the series of longer pieces the death rate is negligible, but in the sixths of young and the sixteenths of old animals it becomes high enough to lessen considerably the value of the data on head frequencies, and in pieces shorter than these it is still higher, so that determination of head frequencies becomes impossible in such pieces.

This increasing death rate with decreasing length of piece is certainly to a considerable extent a consequence of increasing area of cut surface in relation to size of piece. The deaths are practically limited to the first day or two following section. Pieces that survive this period live, with rare exceptions, to the end of the experiment and undergo some degree of reconstitution. In such very short pieces of *P. dorotocephala* the contraction of the cut surface often brings about rupture elsewhere, and contraction at this point produces further rupture in other regions and the piece gradually breaks up. In *P. lata* also the contraction following

TABLE I.

HEAD FREQUENCIES OF FULL-GROWN *P. lata* (11–13 MM.) IN RELATION TO LENGTH OF PIECE AND LEVEL OF BODY.

Length of Pieces.	Number of Animals.	Body Level.	Normal.	Teratophthalmic.	Teratomorphic.	Anophthalmic.	Acephalic.	Dead.	Biaxial Heads.
Fourths.......	210	*A*	99	—	—	—	1	—	—
		B	47	33	—	6	10	4	—
		C	77	16	—	3	3	1	—
		D	96	1	—	1	1	1	—
Sixths........	250	*A*	98	1	—	—	—	1	—
		B	53	30	1	5	9	2	.4
		C	48	33	2	6	8	3	—
		D	43	17	2	8	29	1	—
		E	83	11	1	1	2	2	—
		F	98	1	—	1	—	—	—
Eighths.......	850	*A*	95	1	—	—	1	3	—
		B	73	16	.5	.5	6	4	.6
		C	46	27	1	3	16	7	.8
		D	37	27	1	8	22	5	.7
		E	39	17	2	6	32	3	1
		F	66	11	2	2	16	3	1.6
		G	85	6	—	1	4	4	.9
		H	96	2	—	—	—	2	—
Sixteenths.....	100	*A*	72	1	—	—	—	27	—
		B	61	4	—	1	—	34	1
		C	46	2	—	2	4	46	1
		D	47	8	—	2	8	35	3
		E	39	13	—	1	13	34	2
		F	17	13	—	10	22	38	2
		G	22	9	—	7	21	41	1
		H	29	6	—	2	24	39	4
		I	37	5	—	3	26	29	2
		J	26	7	—	9	28	30	3
		K	34	8	—	11	20	27	6
		L	42	8	1	6	10	37	6
		M	45	13	—	5	5	32	6
		N	47	5	—	4	10	34	6
		O	77	5	—	2	7	9	4
		P	88	5	—	—	1	6	—

section is the chief factor in determining these early deaths in short pieces. In the experiments presented here the pieces dying in this manner are included in the totals in calculating percentages and in graphing, but in certain lines of future investigation it will probably be desirable to exclude these early deaths in determining total head frequencies.

The head-frequency data are tabulated in percentages, the full-grown animals in Table I., the young animals in Table II. From the tabulated data graphs are plotted by the method of assigning numerical values to the different head forms as follows: Normal heads, 5; teratophthalmic, 4; teratomorphic, 3; anophthalmic, 2; acephalic, 1; dead, 0. To obtain a head-frequency value for a given lot of pieces, the number of pieces or the percentage of each head form is multiplied by the numerical value of that form and the sum of these products for the particular lot is divided by the number of pieces in the lot, or, if the data are in percentages, by one hundred. The results are the same whether actual number of pieces or percentages are used. These values plotted as ordinates against the successive levels of section, *A*, *B*, *C*, etc., from the heads of the original animals posteriorly as abscissæ, give curves which permit direct comparison of the head frequencies of pieces of different lengths and from different levels.

TABLE II.

HEAD FREQUENCIES OF YOUNG *P. lata* (4–6 MM.) IN RELATION TO LENGTH OF PIECE AND LEVEL OF BODY.

Length of Pieces.	Number of Animals.	Body Level.	Normal.	Teratophthalmic.	Teratomorphic.	Anophthalmic.	Acephalic.	Dead.	Biaxial Heads.
Fourths.......	150	*A*	90	5	—	1	1	3	—
		B	51	17	—	1	15	16	—
		C	72	15	—	—	7	6	.7
		D	85	3	—	1	3	8	—
Sixths........	150	*A*	91	3	—	—	1	5	—
		B	61	23	1	1	5	—	—
		C	48	34	—	2	9	7	—
		D	48	23	—	1	17	11	.7
		E	69	6	—	1	5	20	.7
		F	95	4	—	—	1	—	—

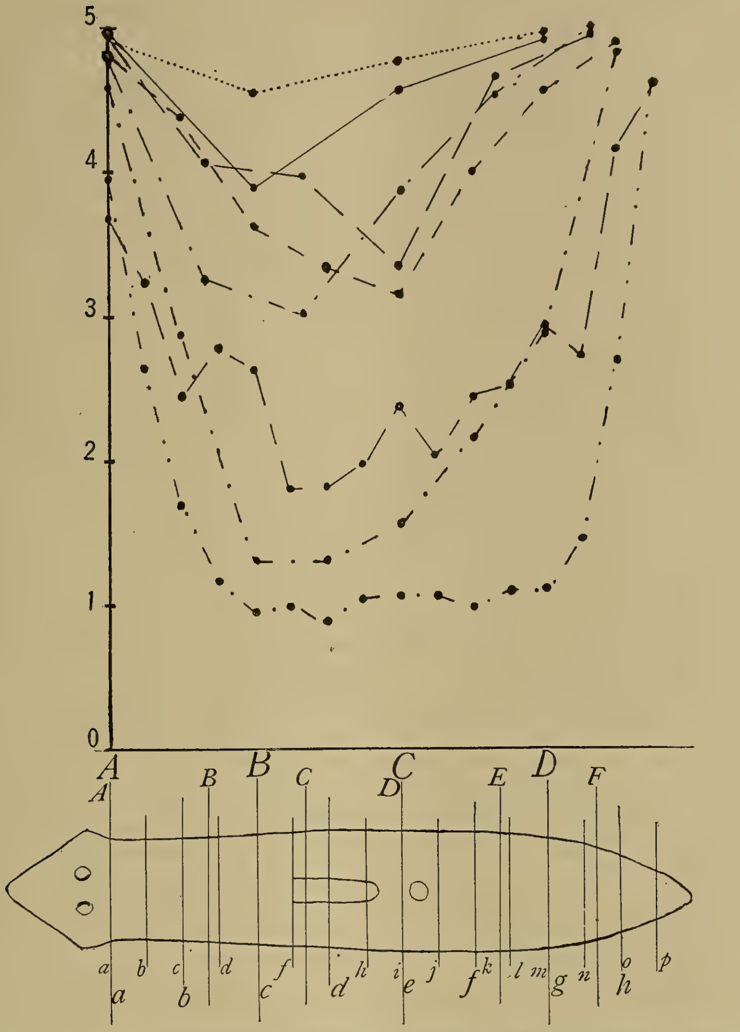


FIG. 21. Head frequencies in relation to length of piece and level of body. *P. lata:* fourths, unbroken line, 110 animals; sixths, long dashes, 250 animals; eighths, short dashes, 850 animals; sixteenths, alternating long and short dashes, 100 animals. *P. dorotocephala:* fourths, dotted line, 110 animals; sixths, alternating dots and long dashes, 110 animals; eighths, alternating dots and short dashes, 70 animals; sixteenths, two dots alternating with short dashes, 60 animals. On the outline of *P. lata* below the graph the levels of section of pieces of different length are indicated: *A–D,* large capitals, fourths; *A–F,* small capitals, sixths; *a–h,* large lower case, eighths; *a–p,* small lower case, sixteenths. The levels of corresponding pieces of *P. dorotocephala* are in part slightly different from those indicated in the diagram since in this species the posterior zoöid is much longer and the pharynx therefore nearer the anterior end in large animals than in *P. lata.*

In the graph, Fig. 21, the data for full-grown animals given in Table I. are plotted, together with head-frequency curves from similar experiments with *P. dorotocephala.* In Fig. 22 the data for young worms from Table II. are graphed in comparison with corresponding data of *P. dorotocephala.*[2] As regards region of body, the data show, both for full-grown and for young animals, that head frequency decreases from the most anterior level of section posteriorly and then increases again with approach to the level of the posteriod zoöid, until at the most posterior level of section it is as high as at the most anterior level, except when lowered by early deaths.

Second, as regards length of piece, the data show for old animals that decrease in head frequency from anterior to posterior region of the first zoöid and increase at levels further posterior becomes greater as length of piece becomes less. The shorter the pieces, the steeper the downward and upward slopes of the curves in Fig. 21. At the most anterior and most posterior levels of the body fourths, sixths, and eighths are practically alike in head frequency, sixteenths somewhat lower anteriorly, but the differences in level of the lowest points of these curves is considerable. The irregularities in the curve of sixteenths are due to the differences in length of the pieces, the variations being, of course, relatively much greater in these very short pieces than in longer pieces. In the young worms (Fig. 22) the differences in steepness in relation to length of piece do not appear in the only data available, those for fourths and sixths, but the sixths show a somewhat lower head frequency than the fourths, except at the posterior end, where it is the same in both.[3]

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[2] The curves of head frequency of *P. dorotocephala* are plotted from data obtained from various sources: fourths, sixths and eighths of old animals and fourths and sixths of young animals from Child ('11*b*, '16, '20*a*), one series of eighths of old animals from Miss M. A. Hinrichs and a complete series of my own for both old and young. All of these data are in general agreement as regards relation of head frequency to length of piece, level of body and physiological age.

[3] It may be noted that the data for young worms are made up in part from animals raised in the laboratory from eggs and in part from animals of the same size similarly raised from cut pieces. The pieces of animals from eggs showed a somewhat higher death rate and therefore a somewhat lower head frequency than those from cut pieces, but the differences were not great.

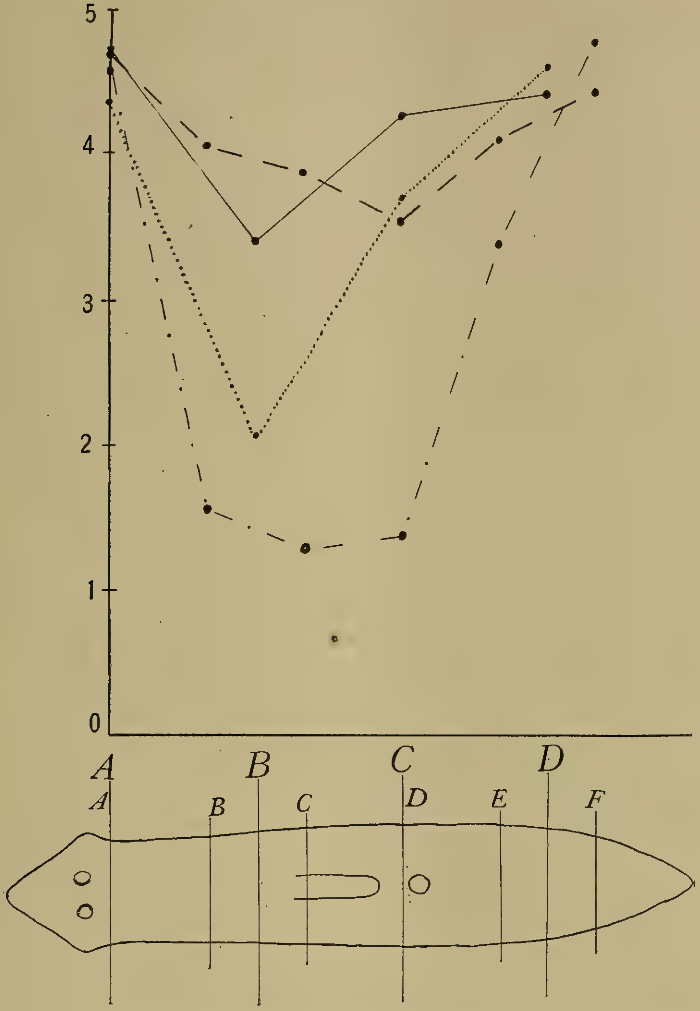


FIG. 22. Head frequencies in relation to length of piece and level of body in young animals. *P. lata:* fourths, unbroken line, 150 animals; sixths, dashes, 150 animals. *P. dorotocephala:* fourths, dotted line, 60 animals; sixths, dots and dashes, 100 animals. The diagrammatic outline below graph indicates levels of section: *A–D,* large capitals, fourths; *A–F,* small capitals, sixths.

Since the number of animals obtained from eggs was limited, it was not possible to determine whether these differences were characteristic or merely the result of slight differences in experimental conditions. For the present, therefore, it has seemed best to combine these data in tabulation and graphing.

Comparison of the curves for *P. lata* with those for *P. dorotocephala* in Figs. 21 and 22 shows that the changes in head frequency with length of piece and level of body are in general of the same sort, but that their range is very much greater in *P. dorotocephala* than in *P. lata*. In full-grown animals (Fig. 21) the head frequencies of fourths of *P. dorotocephala* are somewhat higher than, those of sixths about equal to, and those of eighths and sixteenths far lower than the head frequencies of corresponding pieces of *P. lata* from the posterior regions of the first zoöid, while at anterior and posterior ends of the body the differences between the two species are slight. In the young animals (Fig. 22) fourths and sixths from the posterior region of the first zoöid of *P. dorotocephala* are far below fourths and sixths of *P. lata* from the same region, while at the anterior end of the body the differences are much less, though greater than in old animals.

The graph, Fig. 23, is a comparison of the curves of fourths and sixths of full-grown and young individuals of *P. lata*. This graph shows that the head frequencies in young animals are slightly lower than in corresponding pieces of old animals, but this age difference is much less than in *P. dorotocephala*. Comparison of Figs. 21 and 22 shows that the curves of fourths and sixths of young *P. dorotocephala* are much steeper and fall much lower than those of fourths and sixths of old animals.

Tables I. and II. show the frequencies of biaxial heads, but not of tailless forms. Unfortunately tailless forms were not recorded as such in the earlier experiments. It may be stated, however, that they do not appear in the longer pieces, that their frequency increases with decreasing length of piece, and that, so far as data are at hand, they show no definite relation to level of body. As regards the frequencies of biaxial heads, Tables I. and II. show that they do not occur in the longer pieces, that their frequency increases with decreasing length of piece, and is apparently somewhat greater near the level at which fission occurs than elsewhere.

EXPERIMENTAL ALTERATION OF HEAD FREQUENCY.

In *P. dorotocephala* the head frequencies have been altered experimentally by many different chemical and physical factors (Behre, '18; Buchanan, '22; Child, '16, '20*a*). Moreover, it has been found that head frequencies may be altered in opposite directions in pieces from different levels of the same animals by the

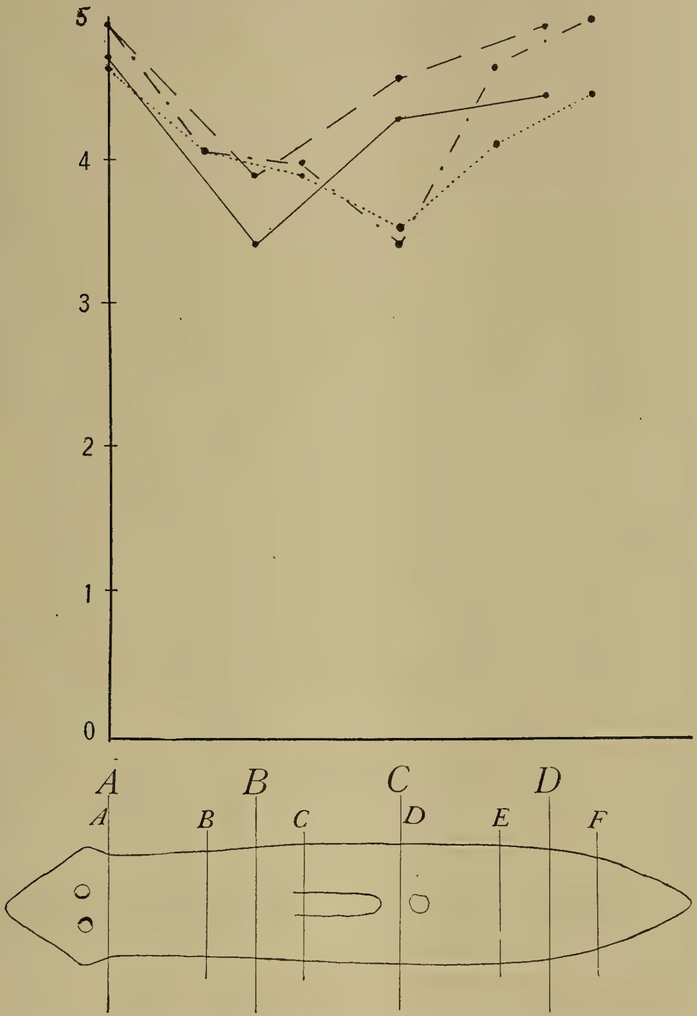


Fig. 23. Head frequencies of fourths and sixths in old and young individuals of *P. lata:* fourths, young, unbroken line; sixths, young, dotted line; fourths, old, long dashes; sixths, old, dots and dashes. The curves in this graph are taken from Figs. 21 and 22.

same concentration of a single agent (Child, '16; Buchanan, '22). In pieces which normally show a high head frequency it may be decreased and in pieces which normally show a low head frequency it may be increased by the same concentration of cyanide or anesthetic. On the basis of these and many other facts concerning the physiology of reconstitution a theory of head frequency has been advanced (Child, '14*a,* '14*d,* '16) which is confirmed by later work (Buchanan, '22). This theory, which is considered more fully below (p. 56), maintains that head frequency in any particular case is determined primarily by the relation between two opposing factors: the one the rate of metabolism in the cells at the cut surface which form the head, the other the rate of metabolism in other parts of the piece. The higher the first in relation to the second, the higher the head frequency; the higher the second in relation to the first, the lower the head frequency. The differential susceptibility of the cells at the cut surface and the other parts of the piece makes it possible through differential inhibition, differential acclimation, and recovery (Child, '20*b,* '21) to alter the relation between the two factors in both directions.

The correlative factor retarding or inhibiting head formation is apparently in part or wholly a matter of nervous stimulation of the cells not directly concerned in head formation. It is most effective during the first few hours after section, when increased CO_2 production, oxygen consumption and susceptibility all show that the pieces are stimulated. In *P. dorotocephala* this stimulation is inhibited and head frequency increased by anesthetics such as ether and chloroform used during a few hours following section (Buchanan, '22), but a day or two later such anesthetics in the same concentrations bring about no increase (Buchanan, unpublished).

Concerning the experiments on *P. lata,* it may be noted, first, that the two factors are concerned in this species, and, second, that apparently nervous stimulation is less effective in decreasing and nervous inhibition in increasing head frequency than in *P. dorotocephala.* Head frequency can be altered in both directions in *P. lata,* but so far as experiments have gone, it appears that general protoplasmic depressants, such as acids, are highly effective, while anesthetics in the stricter sense have little or no effect. These facts are in accord with observations on the living animal, which indicate that the nervous organization is considerably lower than in *P. dorotocephala.*

Another fact pointing in the same direction is that the original polarity can be more readily obliterated and biaxial heads produced by chemical agents than in *P. dorotocephala*. In certain concentrations of acids, for example, the frequency of biaxial heads is much increased.

THE PHYSIOLOGICAL CONDITION OF PIECES FOLLOWING SECTION.

Section of the body results in exposure of a cut surface which gradually contracts and within a few hours cell division and growth begin, giving rise to new embryonic tissue. It has been shown for *P. dorotocephala* that increase in rate of respiration and in susceptibility is slight or inappreciable in fourths or longer pieces, while in sixths and shorter pieces it is marked and increases as length of piece decreases, and also increases from anterior to posterior levels of the first zoöid and decreases again in the posterior zoöids (Child, '14*a*; Robbins and Child, '20; Buchanan, '22; Hyman, '22). Since these changes show definite relations to the polar gradient and are factors in determining head frequency (Child, '14*d*, '16; Buchanan, '22), it is of interest to determine whether similar changes occur and influence head frequency in *P. lata*.

Changes in Susceptibility Following Section.—The data are most readily presented as graphs, plotted by the method used in earlier work (Child, '15*a*, p. 81). This method is briefly as follows: Five stages in the progress of disintegration in KNC or some other agent from intact animals or pieces to completely disintegrated are more or less arbitrarily distinguished and these are given, respectively, the numerical values, 40, 30, 20, 10, 0. In determinations of susceptibility a certain number—*e.g.*, ten—of animals or pieces is placed in the solution used and the number or individuals or pieces in each stage is recorded at hourly or half-hourly intervals. The ordinate of the susceptibility curve for any time is the sum of the products obtained by multiplying the number for each stage by the numerical value of that stage, divided by the number of animals or pieces in the lot. These ordinates are plotted against times in hours as abscissæ.

In the experiments on pieces the susceptibility of the fourths, sixths, and eighths was determined separately for each level of the body; but since the pieces from different levels showed no great differences in susceptibility, the data for all the different levels were brought together in a single curve for each length of piece. Consequently each curve of pieces in the graph, Fig. 24, represents pieces from all levels—*i.e.*, the whole body except the head cut into fourths, sixths, or eighths. KNC *m*/1,000 was used as agent.

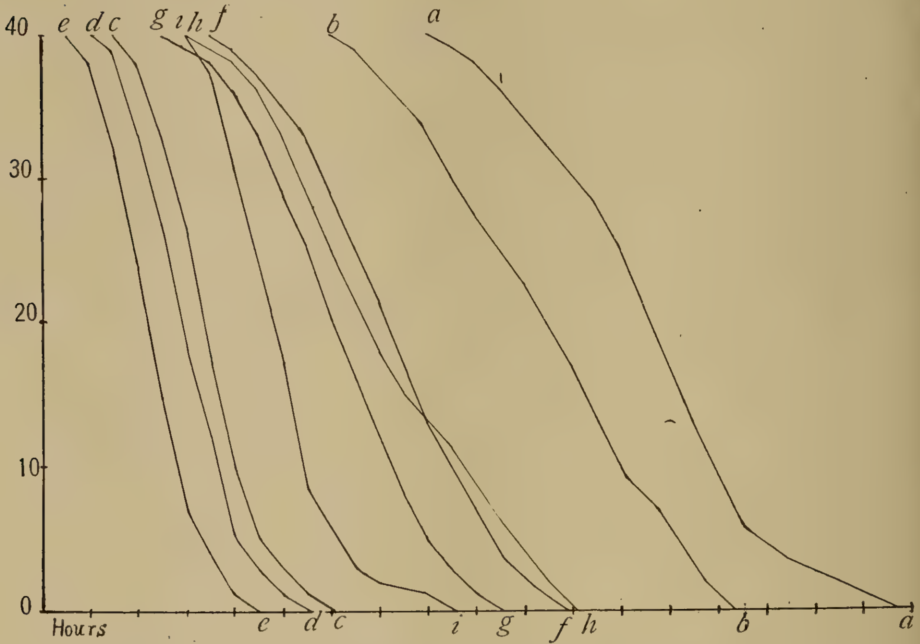


FIG. 24. Graph showing changes in susceptibility following section. Each curve except *aa* and *bb* represents all pieces from 50 animals, *aa*, 50 intact animals, *bb*, 20 headless animals. Further explanation in text.

Fig. 24 shows that susceptibility is greatly increased by section. Uninjured, full-grown animals (*aa*) show the lowest susceptibility of all. Removal of the head increases susceptibility (*bb*). In fourths (*cc*), sixths (*dd*), and eighths (*ee*), immediately after section, susceptibility is greatly increased, and it will be noted that it is highest in eighths, somewhat lower in sixths, and lowest in fourths.

During the first fifteen to twenty hours after section the susceptibility of pieces decreases, then remains nearly stationary for a day or two, and then gradually rises as reconstitution progresses. Curve *ff* shows the susceptibility of fourths left in water fifteen to eighteen hours after section and then placed in KNC; curve *gg*, that of sixths under the same conditions; curve *hh*, that of eighths after forty hours in water. And, finally, curve *ii* shows the susceptibility of young growing animals 5 mm. long raised from pieces—*i.e.*, approximately the susceptibility attained by fourths after reconstitution is completed—that of sixths and eighths being somewhat higher.

Some part of this increase of susceptibility after section is undoubtedly due to the presence of one (*bb*) or two (*cc, dd, ee*) cut surfaces. Other experiments for which curves have been plotted, but which are not shown here, demonstrate that pieces with oblique and therefore larger cut surfaces are more susceptible than pieces with transverse surfaces.

The experiments on susceptibility show one other point of importance which does not directly appear in Fig. 24 and which would require a number of graphs for full presentation. It was stated above that pieces of the same length from different levels show approximately the same susceptibility. In the section on susceptibility gradients it was shown that susceptibility decreases from the anterior to the posterior end of the first zoöid and increases again with approach to the level of the posterior zoöid. Even if the susceptibility of pieces from all levels were approximately the same immediately after section, it would be evident that the increase in susceptibility must have been much greater in pieces from posterior than in those from anterior levels of the first zoöid and from the posterior zoöid. It follows from this fact that susceptibility is not simply a matter of the presence of cut surfaces, but depends in part upon level of the body from which pieces are taken.

Comparison of these changes in susceptibility following section with those in *P. dorotocephala*[4] brings to light some interesting physiological differences between the two species. In the first place, removal of the head does not appreciably increase susceptibility in *P. dorotocephala*. In fourths it is increased only slightly,

[4] See Child, '14*a*. His results have been repeatedly confirmed by myself and by other students in laboratory experiments.

in sixths somewhat more, and in eighths still more, but in all cases much less than in *P. lata*. Moreover, during the first twenty-four hours after section it usually decreases almost or quite to the same level as that of whole animals. In short, the presence of one or even two cut surfaces has in itself little or no effect in increasing susceptibility in *P. dorotocephala*. Differences in body level are far more important, particularly in the shorter pieces. Even in sixths or less the increase is slight in anterior pieces, becomes greater toward the posterior end of the first zoöid, and is again slight in the posterior zoöid.

Evidently the increase in susceptibility in relation to cut surfaces and the differential increase at different levels of the body depend, at least in part, on different factors. The former, which seems to be essentially a cellular wound reaction, followed by cell division and growth, is the more conspicuous feature in *P. lata*. The differential increase, on the other hand, is more conspicuous in *P. dorotocephala*, but is present also in *P. lata*, and appears to be a stimulation of the piece as a whole. Various facts, such as its short duration and its inhibition by anesthetics (Buchanan, '22), indicate that it is nervous in character. Moreover, it is of interest to note that the differential increase varies inversely as susceptibility at different levels of whole animals and inversely as head frequency at different levels, while the increase in relation to cut surfaces varies directly with area of cut surface in relation to size of piece.

Changes in Rate of CO_2 Production Following Section.—Colorimetric estimations of CO_2 production confirm the data on susceptibility as indicative of changes in rate of respiration. In each of these experiments five 12–13-mm. headless animals entire were compared with five headless animals cut into eighths. The starting point was pH 7.9, and the pH was recorded at regular intervals, and the time required to reach pH 7.3 was also determined. After the experiment both lots were weighed, and in all cases the weight of the pieces was less by some 20 to 50 per cent. than the weight of headless animals, because some loss of intestinal contents, fluids, or even cells occurs when pieces are cut. In all cases, however, the rate of decrease of pH of the pieces was equal to or higher than that of the entire headless animals. In Table III. the length of time required for change from pH 7.9 to pH 7.3 is given in hours and minutes for ten milligrams of headless animals and pieces, as calculated from the actual weights and times. This method of presentation is open to certain objections, but has the advantage of brevity and clearness. Of the nine experiments in Table III. five were with headless animals and eighths immediately after section, four with headless and eighths twenty-four hours after section. In every case the time is much less for the pieces, but is somewhat greater after twenty-four hours than immediately after section. These data confirm the susceptibility data in showing that rate of respiration in eighths immediately after section is greatly increased over that of headless controls, and that after twenty-four hours it is somewhat lower, but still far above that of the controls.

TABLE III.

TIME IN HOURS AND MINUTES REQUIRED FOR CHANGE FROM pH 7.9 TO 7.3 CALCULATED FOR 10 MG. FROM ACTUAL WEIGHT AND TIME.

	Headless.	Eighths.	
		Immediately after Section.	24 Hours a. s.
	10:36	4:10	——
	8:49	4:57	——
	10:30	——	4:10
	12:20	3:42	——
	12:40	——	9:20
	6:12	——	5:12
	10:40	4:53	——
	8:30	6:34	——
	7:22	——	4:44
Mean,	9:48	4:51	5:51

Changes in Oxygen Consumption Following Section.—Table IV. gives the results of determinations of oxygen consumption made by Dr. Hyman. In the first experiment wholes, headless, and pieces are compared, in the others only headless and pieces, the headless animals being used because they show less motor activity than wholes. The pieces include both sixths and eighths from all levels posterior to the head. Table IV. shows that oxygen consumption is greatly increased in pieces immediately after section, as compared with headless animals, and in the first experiment in headless animals as compared with wholes. Twenty-four hours after section the oxygen consumption is lower in some cases, higher in others, than immediately after section, and increase is more frequent in the pieces than in headless animals. Pieces of *P. dorotocephala* also show a similar increase in oxygen consumption in some cases (Hyman, '22). In this respect the data on oxygen consumption apparently disagree in part with those on CO_2 production and susceptibility. The reasons for this apparent disagreement are not as yet certainly known, but it seems probable that nutritive condition and growth at the cut surfaces which is appreciable within twenty-four hours are the factors chiefly concerned.

Table IV.

Oxygen Consumption of *P. lata:* Whole Animals, Headless, and Sixth and Eighth Pieces Immediately and Twenty-four Hours after Section. Calculated in Cubic Centimeters of Oxygen Consumed per Gram in Four Hours.

Calculations at 20° C.

Experiment.	Wholes.	Headless.		Pieces.	
		Immediate.	24 Hours.	Immediate.	24 Hours.
1........	1.31 ——	1.40 ——	1.30 ——	1.93 2.11	1.68 1.78
2........	——	1.06	.99	1.44	Lost.
3........	——	1.34	1.47	1.60	1.83
4........	——	1.26	1.11	1.51	1.71
5........	—— ——	1.00 ——	1.20 ——	1.59 1.73	1.79 2.18

Time of Head Determination.

It has been shown that in *P. dorotocephala* the head frequency characteristic of a certain length of piece at a certain level is determined within a few hours after section to such an extent that it is only slightly or not at all altered by decreasing the length of piece after that time (Child, '14*d*). The experiment to test this point gives essentially the same results in *P. lata,* except that the differences in head frequency in long and short pieces are less than in *P. dorotocephala.* Fig. 25 shows the pieces used in the experiment. A large number of long pieces are cut with anterior ends at level *aa* (Fig. 25), and at intervals the anterior ends of a certain number of them—*e.g.,* fifty—are cut off as short pieces at the level *bb* and their head frequencies recorded after reconstitution. Omitting tables and graphs, the most important results obtained are as follows: In the short pieces isolated at once in the usual manner the chief head frequencies are: normal, 40 per cent.; teratophthalmic, 17 per cent.; acephalic, 30 per cent. In short pieces cut from anterior ends of long pieces after five hours head frequencies are: normal, 80 per cent.; teratophthalmic, 6 per cent.; acephalic, 2 per cent. In short pieces cut in the same way after twenty-three hours 100 per cent. normal heads appeared.

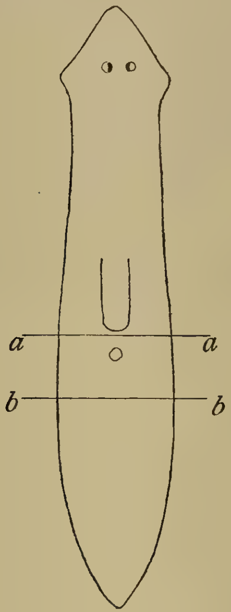
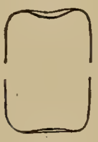


FIG. 25. Outline indicating levels of section, *aa* and *bb,* in experiment on head determination.

This experiment shows that under ordinary conditions factors determining head frequency are to some extent effective during the first few hours after section, for during this period the alteration of head frequency characteristic of long pieces by great decrease in length of piece becomes progressively less. This, of course, does not mean that head frequency can not be altered in other ways after this time; it merely means that conditions determining head frequency are to some extent established during this time. Since this is the period of greatest stimulation of pieces, since that stimulation is greater in shorter than in longer pieces and in posterior than in anterior pieces of an individual or zoöid, and since decrease of that stimulation during the first few hours after section increases head frequency, it appears highly probable that the differential stimulation of pieces after section is a factor in determining that under ordinary conditions head frequency is lower in shorter than in longer and in posterior than in anterior pieces of individual or zoöid.

Rate of Growth and Differentiation and Regional Differentials in Development at Anterior Cut Surfaces.

Only a brief statement of general results along these lines is given at the present time. The growth reaction at anterior cut surfaces is much more rapid in *P. lata* than in *P. dorotocephala.* In the former the strong contraction following section has very largely disappeared and a distinct outgrowth of new tissue is present over the whole surface twenty-four hours after section (Fig. 26), while in the latter the cut surface is still strongly contracted and there is less than half as much new tissue (Fig. 27). On posterior surfaces the differences between the two species are less marked (Figs. 26, 27). Two days after section the differences between the species remain about the same, but during the third day the rate of growth in *P. dorotocephala* becomes more rapid, as compared with that in *P. lata,* and on the fourth day the amounts of new tissue are apparently about the same. Evidently the initiation and acceleration of growth at the cut surface occurs



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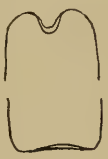

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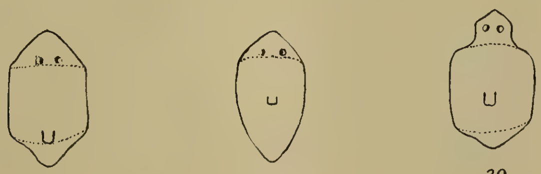
Figs. 26, 27. New tissue twenty-four hours after section: Fig. 26, *P. lata;* Fig. 27, *P. dorotocephala.*

earlier in *P. lata* than in *P. dorotocephala.* This difference probably accounts, at least in part, for the fact that susceptibility and rate of respiration in pieces of *P. lata* remain considerably higher after section than in whole animals, while in *P. dorotocephala* the decrease within twenty-four hours after section is greater, often to the level of whole animals.

The rate of growth and of differentiation of the head, as determined by the time at which the eyes become visible, is approximately the same in anterior regions and in the posterior zoöid in both species, and in both it is more rapid in these regions than at posterior levels of the first zoöid. It also decreases and the differences in rate at different levels increase with decreasing length of piece. In other words, curves of rate of differentiation of head plotted from repeated observations of developing pieces resemble in their relations to body level and length of piece the head-frequency curves. Comparison of the two species shows, however, that in the shorter pieces and the more posterior levels of the first zoöid the rate of differentiation is slightly lower in *P. lata* than in *P. dorotocephala.* That is to say, in *P. lata* the head frequency in such pieces is higher, but the rate of differentiation of the head is lower than in *P. dorotocephala.*

As regards the portion of body posterior to the head which is formed by new tissue, there is little difference at different levels of section in longer pieces, but with decreasing length of piece this differential appears. In eighths, for example, from levels near the anterior end of the animal and near or in the posterior zoöid, the eyes develop at or slightly anterior to the boundary between new and old tissue (Figs. 28, 29), while in pieces from the poste-


28 29 30

FIGS. 28–30. Regions of body formed by anterior new tissue at different levels in eighths of *P. lata:* Fig. 28, anterior region of first zoöid; Fig. 29, posterior zoöid; Fig. 30, posterior region of first zoöid.

rior region of the first zoöid—*i.e.*, about the level of the pharynx—a considerable portion of the body posterior to the eyes develops from new tissue (Fig. 30). A similar regional differential appears in shorter pieces in *P. dorotocephala* and *P. maculata*, but the amount of difference differs somewhat in the different species. In *P. foremannii* and other species which possess no posterior zoöid the regional differential continues to change in the same direction to the posterior end of the body—*i.e.*, the more posterior the piece, the longer the portion of the body formed from new tissue at the anterior end (Morgan, '01).

This regional differential is evidently determined by a complex of factors—*e.g.*, rate of growth of new tissue, size of new head, degree of inhibition in its development by stimulation of the piece, rapidity of reorganization of old parts, and perhaps others. The chief point of interest at present, however, is the fact that this regional differential shows the same relation to body level and length of piece as does head frequency. Its relation to the polar axial gradient is therefore evident. Moreover, experiments show that this differential can be altered and controlled by the same factors by which head frequency is altered and controlled.

Discussion.

Reconstitution in Relation to Body Level, Length of Piece, and Physiological Age.—It is evident that the axial susceptibility gradient is an indicator of fundamental physiological differences along the axis and many facts indicate that such differences are primarily quantitative rather than qualitative. Head frequency, differential increase in susceptibility following section, rate of growth of new tissue at the anterior end, and the portion of the body posterior to the eyes which is formed by new tissue all show a gradation according to body level and therefore a definite relation to the susceptibility gradient. This is true for *P. dorotocephala* as well as for *P. lata*. That the physiological factors which determine these graded differences in reaction to section and in the processes of reconstitution are fundamentally quantitative, not qualitative, is indicated by the fact that there is no evidence of fixity or specificity in their relation to body level. All the features of reconstitution characteristic of a given body level under normal or standard external conditions can be altered to those characteristic of other levels by changes in external conditions which are primarily non-specific and quantitative in physiological effect. This has been shown for *P. dorotocephala* in many ways and is also true for *P. lata,* though only a part of the evidence appears in the present paper. It has also been shown for *P. dorotocephala* that the susceptibility gradient is an indicator of a corresponding gradient in rate of respiration (Robbins and Child, '20; Hyman, '22), and the data on the changes in susceptibility and rate of respiration in pieces following section leave no doubt that a similar relation between susceptibility and respiratory rate exists in *P. lata.* If this is true, the inference is justified that the reconstitutional differences at different levels are in some way associated with the differences in rate of metabolic reactions, as indicated by rate of respiration.

The relations of reconstitutional processes to length of piece also appear to be non-specific and quantitative in character, for they, too, can be altered by the quantitative action of external factors. And, finally, the relation of head frequency to physiological age is apparently non-specific and quantitative and can be altered experimentally by changes in condition which affect primarily rate, rather than kind of metabolic reactions.

Nowhere do we find any evidence for the existence of specific formative substances. Given the specific protoplasm of a planarian species, the differences in the reconstitutional processes and results are apparently dependent primarily upon quantitative dynamic differences rather than upon specific qualitative factors.

Physiological Analysis of Head Frequency.—It has been shown that the head forms differing from the normal which appear in the reconstitution of *P. dorotocephala* represent various degrees of differential inhibition of head development, and that they can all be produced by chemical and physical agents, as well as by physiological factors (Child, '16, '20*a,* '21; Behre, '18; Buchanan, '22). All the experimental evidence supports the conclusion that two antagonistic factors are concerned in the reconstitution of a head—the one positive or determining, the other negative or inhibitory—and that head frequency in any particular case depends on the relation between these two factors (Child, '14*a,* '14*d,* '16, '20*a,* '21;

Behre, '18; Buchanan, '22). The evidence indicates further that the positive determining factor is the rate of activity of the cells at or near the cut surface (Fig. 31, x) which react to section by dedifferentiation, division, and growth, and are directly concerned in head formation. The inhibiting factor, on the other hand, is apparently the correlative influence on x of other parts of the piece (Fig. 31, y) which tends to retard or inhibit the dedifferentiation and growth of the x cells. More or less excitation of the region y occurs temporarily after section, probably largely because of the injury to the nerve cords, and experiments have shown, first, that head frequency decreases as the degree of this excitation increases—*e.g.*, at the more posterior levels of the first zoöid and in shorter pieces—and, second, that inhibition of this excitation increases head frequency. It has been shown further that the differential susceptibility of the regions x and y and the different degrees of excitation of y at different levels of the body provide a physiological basis for altering head frequency experimentally in either direction with the same concentration of a single chemical agent (Child, '16) and with many different agents and conditions (Child, '20*a*; Behre, '18; Buchanan, '22). In short, the facts indicate that head frequency varies directly with rate of metabolism in x and inversely with rate in y. This relation has been stated in the following brief form:

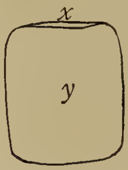


FIG. 31. Diagrammatic outline of piece after section: x, region directly concerned in formation of head; y, region of correlative inhibitory effect on head development.

$$\text{head frequency} = \frac{\text{rate } x}{\text{rate } y}.$$

This formula is perhaps not complete, but serves provisionally to indicate the opposite relation of the two factors to head frequency. If it indicates the relation correctly, it follows that head formation in reconstitution really takes place in spite of the rest of the piece.

In other words, in so far as the x cells become independent of y, they dedifferentiate and begin the development of a new individual, the head arising first as in embryonic development. The various differentially inhibited head forms result from different degrees of inhibition of x by y or by some external factor.

All the experimental evidence at hand indicates that this interpretation holds for *P. lata* as well as for *P. dorotocephala,* the chief difference being that in *P. lata* the inhibiting action of y shows less increase with decrease in length of piece and at more posterior levels of the first zoöid, and that head frequency is therefore normally higher in the shorter pieces and at more posterior levels of the first zoöid and is less increased by inhibition of y in *P. lata* than in *P. dorotocephala.* This difference between the species is what we should expect if the inhibiting action of y on head formation is nervous in character, as the facts lead us to believe. The differences in excito-motor behavior and in development of sense organs certainly indicate a lower degree of nervous organization in *P. lata* than in *P. dorotocephala.* Moreover, the differences in degree of excitation of y following section and in head frequency in relation to level of body and length of piece are less in *P. lata* than in *P. dorotocephala,* and this again suggests a lesser degree of specialization of different body levels in relation to the axial gradient.

The fact that in *P. lata* head frequency is almost as high in young as in old animals, while in *P. dorotocephala* it is much lower in young than in old, also indicates that the region y is less effective in inhibiting head formation at x in *P. lata.* Rate y is higher in relation to rate x in young than in old animals because the tissues of young animals have in general a higher rate of metabolism than those of old, but this difference has much less effect on head frequency in *P. lata* than in *P. dorotocephala.* The more rapid growth reaction in *P. lata* also indicates that y is less effective in this species in inhibiting dedifferentiation and growth of x. The physiological analysis of reconstitution leads, in fact, to the same conclusion as observations on the behavior of the two species, viz., that *P. lata* is a more primitive, a less highly specialized form than *P. dorotocephala.*

Tailless Forms and Biaxial Heads.—Tailless forms develop from very short pieces in which a single polarity, in these experiments the original polarity, is maintained. They arise when the cells at the posterior cut surface are not sufficiently active in relation to parts anterior to them to grow at the expense of the latter. Heads cut off immediately behind the eyes always remain tailless, and in general when the piece is so short that the posterior cut surface is very close to the new head the development of a new posterior end is inhibited, because the rate of metabolism at other levels of the piece is so high that the cells at the posterior end can obtain but little nutrition.

Biaxial forms arise in very short pieces of *Planaria* when a new physiological gradient arises in relation to the posterior cut surface. In the short piece conditions are most favorable for the origin of such new gradients because there is but little physiological difference between the two cut ends—*i.e.,* these short pieces are nearly apolar because they are short, consequently each cut surface may become a dominant region and determine a polarity in the opposite direction to the other (Child, '15*b,* pp. 98–100). The higher frequency of biaxial heads under ordinary conditions in *P. lata* than in *P. dorotocephala* suggests that polarity—*i.e.,* the longitudinal axial physiological gradient—is less stable in the former, and this also is in accord with the conclusion that *P. lata* is a less specialized form than *P. dorotocephala.*

SUMMARY.

1. *Planaria lata* possesses a short posterior zoöid and undergoes fission. Fission does not interfere with sexual maturity, probably because the level of fission is far posterior to the genital pore.

2. Susceptibility decreases from the anterior to the posterior end of the first zoöid and increases again with approach to the posterior zoöid.

3. Head frequency varies in relation to level of body in the same way as susceptibility. The differences in head frequency at different levels increase as length of piece decreases, and head frequency is slightly lower in young than in old animals.

4. Isolation of pieces is followed immediately by a great increase in susceptibility, CO_2 production, and oxygen consumption, then a gradual decrease occurs during 12–24 hours to a level still far

above that of whole animals. Later a gradual increase coincides with the progress of reconstitution.

5. The increase in susceptibility after section in relation to body level and length of piece varies inversely as the susceptibility gradient of whole animals and the head frequency in reconstitution.

6. The factors determining whether a piece shall or shall not develop a head become to some extent effective within a few hours after section.

7. The experimental data all support the conclusion that in *P. lata,* as in *P. dorotocephala,* two factors are concerned in determining head frequency: one the rate of metabolism in the cells at the cut surface, being positive or determining; the other the rate of metabolism in other regions of the piece, being negative or inhibitory. Head frequency in any particular case is determined by the relation between these two factors. Apparently the inhibitory factor is less effective in the shorter pieces and at more posterior levels in *P. lata* than in *P. dorotocephala.*

8. The physiological analysis of reconstitution agrees with observations on behavior in indicating that *P. lata* is a less highly specialized form than *P. dorotocephala.*

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CONTENTS

McNAIR, GEORGE T. *Motor Reactions of the Fresh-Water Sponge, Ephydatia fluviatilis* 153

ALLEE, W. C. *Studies in Marine Ecology.* I, *The Distribution of Common Littoral Invertebrates of the Woods Hole Region.* 167

LAMBERT, W. V., RICE, W. S. AND WALKER, H. C. A. *Food and Parthenogenetic Reproduction as Related to the Constitutional Vigor of Hydatina senta* 192

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BIOLOGICAL BULLETIN

MOTOR REACTIONS OF THE FRESH-WATER SPONGE, *EPHYDATIA FLUVIATILIS*.[1]

GEORGE T. McNAIR,

Hull Zoölogical Laboratories, The University of Chicago.

Parker's work on *Stylotella heliophila* ('10) has suggested the possibilities of similar experiments with fresh-water sponges and a comparison of the reactions of the two types.

Aside from Parker's monograph practically nothing has been published on the motor reactions of sponges. Dr. R. E. Grant (1825–'26) published a series of papers on the "Structure and Functions of Sponges," which are of historical interest only. He quotes an earlier paper (Ellis and Knight, *Transactions of the Royal Society of London*, 1765), in which these men stated that they had seen the orifices on the surfaces of sponges contract and dilate themselves. Grant studied a large number of salt-water species, both in their natural habitat and in the laboratory. He was the first to notice and accurately describe the currents of water coming from the fecal orifices (oscula) and spoke of them as mysterious currents because he could not learn what caused them. He irritated the orifices and other tissues with corrosive acids, red hot wires, etc., but under no circumstances did he see the osculum closed and erroneously concluded that there was a complete lack of irritability and contractility, attributing the statements of Ellis and Knight concerning the "systole and diastole of the fecal orifices" to "some optical deception a little assisted by the imagination."

Parker made two types of experiments on *Stylotella*, noting the responses of the oscula, ostia, and choanocytes first to mechanical and then to chemical stimuli. He studied the reactions of the sponge to flowing water, injuries, and cuts. Likewise he studied the effects which ether, chloroform, cocaine, strychnine, and atropine produced upon the sponge and compared his results with the known effects of these drugs on smooth muscle and on ciliated tissue.

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[1] The problem was suggested to me by Dr. W. J. Crozier. It is with great pleasure that I acknowledge my indebtedness to him and also to Professors C. M. Child and W. C. Allee for their helpful suggestions and criticisms.

Ephydatia fluviatilis (L.), the fresh-water sponge with which the experiments described in this paper were made, differs so greatly in structure from *Stylotella* that it was impossible to duplicate many of Parker's experiments. The responses of the oscula were watched to determine the effects of various mechanical stimuli such as injuries, electrical stimulation, and changes in temperature. The purpose was to determine the effects of such stimuli and the rate of possible transmission of these effects from one part of the body to another.

All the sponges used in the following experiments were collected in small ponds along the railroad tracks in the neighborhood of Hammond and Buffington, Indiana. They were carried to the laboratory in fruit jars and then transferred to large jars of slowly running water.

The ponds from which the material was collected[1] were shallow, often almost filled with bulrushes. The bottoms were covered with mud and cinders. The sponges were, for the most part, growing on the under side of old railroad ties floating in the water and were more or less cushion-like in form and without much branching. Some, however, were found growing on the submerged parts of plants or on leaves, but here their form was more spreading, following that of the body to which they were attached.

The body of the sponge is dotted all over with very small dermal pores or ostia. The oscula are relatively prominent little chimneys of dermal membrane, standing our from almost any point on the suface. Bundles of smooth, pointed, and almost straight silicious spicules make up the skeleton. The internal canals of the body meander irregularily between these bundles of spicules. The dermal membrane, which completely envelops the body, is held up in tent-like elevations by the protruding ends of the spicules.

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[1] See Shelford, "Animal Communities in Temperate America," Chapter VIII.

The oscular chimneys seem to be kept open by the pressure of the currents of water passing out through them. They are continuously changing their form. At times one will be long and slender; again it will be shorter and much thicker or even dome-like, the base being as much as three or four times the height. In instances of the first type the mouth of the chimney will probably be open almost to the width of the diameter, although these mouths are frequently seen to be nearly closed. In the type last mentioned they are usually small.

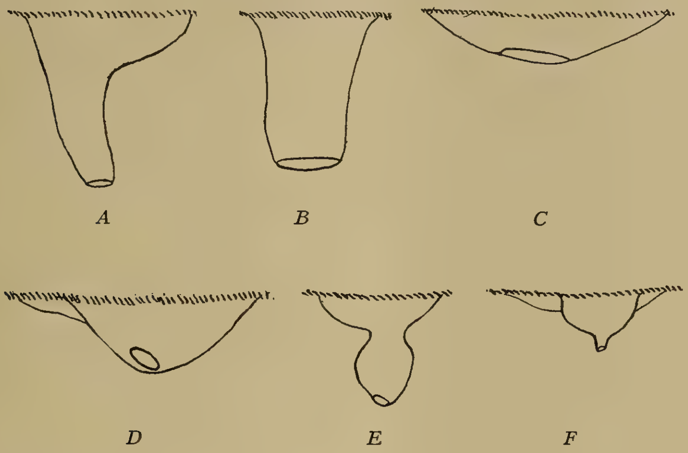

FIG. 1. Various forms in which the same oscular chimney was observed.

These variations in shape lead to the belief that the entire chimney contains sphincter-like bands. If all such bands were about equally contracted the result would be a long slender chimney, Fig. 1, *A* and *B*. If only those at the end contracted and the others relaxed, the result would be the dome-like shape, Fig. 1, *C* and *D*. Several instances of a contraction of the terminal sphincter and also of the chimney near its base, with a relaxation between, were noticed. The result was a sort of globe-like chimney attached to the sponge body by a narrow neck, Fig. 1, *E*. The chimneys also seem capable of a decided shortening without much change in diameter. This may be brought about by a contraction of longitudinal fibers, but it could not be definitely demonstrated.[1]

Effects of Currents of Water on the Osculum.

Potts ('87) makes the statement that he found *E. fluviatilis* in both quiet and running water. It seems to grow best in ponds of quiet water, but very fine specimens are found on the under side of rocks in rapids or at the base of water falls where the river current is the swiftest.

To get an idea of the effects of water currents, a few sponges were placed in a flat dish containing about a quart of water and left over night. The next morning the oscula were still expanded, and, by watching the movements of tiny particles of suspended material in the water, steady currents could be seen flowing through them. This test was repeated with the same results. It was noticed, however, that if the sponges were left in such a dish of unchanged water much longer than a day they gradually became less active and in many cases soon died.

To determine the effect of a swift current, a glass tube was connected with the water tank and placed in one side of a dish containing the sponges. The water was turned on at full force and left running for about four hours. After transferring the sponges to a watch glass for observation (without taking them from the water) it was found that the entire chimney had contracted until it was almost flat, but that the mouth of the osculum was wide open and currents of water were coming out. The following illustration shows the extent of the contraction of the chimney.

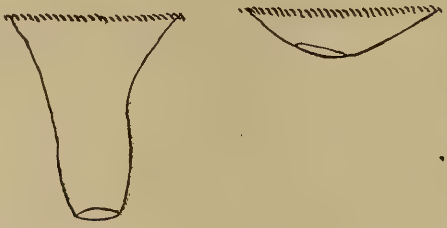

FIG. 2. A chimney before and after a strong current of water had been applied, showing the extent of the contraction.

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[1] See, E. A. Minchin, Lankester's "Treatise on Zoölogy," p. 44.

Within thirty minutes after taking the sponges out of the swift current the chimney had again extended to about half of its original length. The osculum was slightly contracted.

The current had caused the sponges to roll and tumble over the bottom of the dish to some extent. To find out if this had been the cause of the shortening of the chimneys, the sponges were placed in watch glasses which, in turn, were placed within the larger dish. When the water was turned on, the force of the current against the curvature of the watch glass held the sponges in place. This time a swift current flowed over the sponges but they did not roll about. Within twenty minutes the chimneys had flattened out as before.

The sponges were now placed in a large jar in which a slow current of water was kept going. After being there for a few hours the chimneys were found to be well extended and with strong currents coming out through them.

Neither the absence of currents nor the presence of strong currents causes the osculum to close although the latter does cause a general shortening of the oscular chimney. In small quantities of water, however, the sponges soon became less active and in time died; the smaller the quantity of water the sooner the changes could be noticed. This effect could not have been due to the absence of the mechanical stimulus of flowing water, because if placed in a large tank of quiet water the sponges will live for a long time. Nor could it have been caused by lack of oxygen, because the dishes which were used were large and shallow so that the water was well exposed to the air. It must have been produced then by self-poisoning from the products of metabolism that accumulated in the small quantity of water. The greater general vitality of the sponges when placed in slow running water or in large tanks of quiet water is accounted for by the fact that these products were removed as they were thrown off by the sponges or were diffused through the large volume of water.

These results are entirely different from those obtained by Parker from somewhat similar tests with *Stylotella*. When he transferred *Stylotella* from the natural habitat to tanks of quiet sea water, the oscula invariably closed within ten minutes and did not reopen until they were placed in running water. He was able to prove that the mechanical stimulus of the currents of water on the outlet tip of the finger where the osculum was located, caused it to open. The oscular closure seems to be a protective adaptation of *Stylotella,* which is a shallow salt-water species frequently left exposed to the air when the tide is out. Since the fresh-water sponge is never exposed to such tidal conditions, one would not expect to get the same reactions.

Brushing.

Rubbing the sides of the chimney with a needle, prodding it gently, or even inserting the needle into the mouth of the osculum and rubbing the inside of the chimney, caused no noticeable reaction; however, if the needle were carefully rubbed around the edge of the mouth of the osculum, an immediate contraction of the orifice would follow and it would continue to contract for about three minutes. Following the contraction of the sphincter at the orifice a wave of contraction would travel down the chimney although the amount of contraction would be less than at the orifice. On a chimney of 1.5 mm. length it would require about six seconds for this wave to run from the tip to the base of the osculum. In the next three or four minutes it would again expand to its original size. The edge of the mouth of the osculum, therefore, seems to be more sensitive than the rest of the chimney. Concerning the reactions of *Stylotella,* Parker states that stroking an open osculum with a bristle or brush does not cause it to close, but if it is closed, this treatment may at rare intervals induce it to open.

Sharp Blow.

Several times while getting the electrodes into position to work with the inductorium one of the wires of the electrode would catch on a spicule and, springing loose, strike an oscular chimney. Each time the entire chimney would shrivel up immediately in a more or less collapsed condition, but within twenty or thirty minutes it would be open and functioning as before.

Cutting.

Cutting into the body seemed to have no effect in the way of induced movements. With a small scalpel an incision was made into the body of the sponge 3 mm. from the base of the chimney without causing any change in the rate of the currents or in the size of the chimney. Another cut right at the base of the chimney caused it to close. It did not reopen for two days. This cut was so near the chimney, however, that the closing was probably due to cutting into the chimney cavity and when this had healed over the chimney again expanded. The result of such injuries differs from the effect of similar ones on *Stylotella*, where a cut from 3 mm. to 5 mm. from the osculum was found to cause closure within nine minutes.

Pin Sticking.

Sticking a needle into the flesh of the sponge at a distance of 2 mm. from the osculum caused no noticeable effect on the osculum. The same type of injury 5 mm. from the osculum of *Stylotella* resulted in the osculum closing within ten minutes and remaining closed for several hours.

Exposure to the Air.

A sponge was lifted from the water and held in the air for three minutes. A large chimney was noticeable before, but could not be found after placing the sponge back in the water; neither could it be found at any later time.

Thermal Responses.

The average temperature of the water in the tanks in which the sponges were kept was about 27° C. Eight of the sponges which had well developed oscular chimneys and from which strong currents of water were flowing were placed in dishes in a refrigerator which had a uniform temperature of 5° C. and left until the water had been cooled to 7° C. They were examined at regular intervals. At 16° there was no noticeable change in any of the sponges. At 11° all had a shriveled appearance, some of the oscula were open slightly, and very slow currents coming from them were barely visible. At 10° no oscula could be found, and at 7° the dermal membrane had completely shrunk in over the body of all the sponges and no sign of activity could be detected. The dishes were transferred to an incubator and observations were made until the temperature of the water had reached 40° C. At 24° the dermal membranes had taken on their usual appearance, the oscula were somewhat expanded and currents were issuing from them. At 34° the chimneys were more extended and stronger currents were coming from them than at any previous time. At 37° they were still expanded but were slightly flabby and the currents were weaker. At 40° the oscula had all disappeared, the dermal membranes were completely shriveled. None of the sponges recovered after being raised to the temperature of 40°.

Comparing these results with those obtained by Parker, it will be seen that the salt-water sponge is apparently less affected by temperature conditions than is the fresh-water species. With *Stylotella* at between 9° and 10° the oscula and ostia remained open and the only effect seemed to be that the currents became slow. At 40° there was a slight constriction of the osculum and the currents gradually became slower and stopped. At 45° there was a flabby contraction of the oscula and the currents ceased abruptly. They did not recover after being raised to the temperature of 45°.

LIGHT.

Neither full sunlight nor complete darkness seemed to have any effect on *E. fluviatilis*. One large, healthy specimen was placed in a dark cupboard and left there four days. At the end of this time the green color due to contained algæ had faded, but the activity of the sponge in producing currents had not diminished in any way.

ELECTRICAL STIMULATION.

By using an inductorium with electrodes of fine platinum wires, the effect of weak Faradic stimulation could be observed. The electrodes were applied to three different places: first, on the side of the chimney; second, at the base of the chimney, one wire on each side; third, at points on the flesh immediately back of the chimney at distances of from 3 mm. to 5 mm. from it. A weak stimulus when applied to the tip of the chimney caused a contraction of the oscular sphincter and a gradual wave of contraction down the entire length of the chimney. This wave traveled down at the rate of about 1.5 mm. in four seconds. A strong stimulus applied to the chimney caused a complete relaxation, as though paralyzed, and from which it did not recover.

STIMULATION AT THE SIDES OF THE CHIMNEY.

Choosing chimneys which were long and well extended the electrodes were applied at one side of the chimney, Fig. 3, *A*. When a weak stimulus was used, the response required about one minute before it appeared. A mild stimulus resulted in an immediate response. First, the chimney would bend toward the electrodes, the bending occurring at the points where the wires touched the chimney, Fig. 3, *B*. This bending always occurred within thirty seconds from the time the stimulus was started. At the same time, but continuing longer, the entire chimney would shrink slightly. This slow shrinking continued for from one to five minutes. If the wires were applied to the opposite side (Fig. 3, *C*) while the chimney was still bent as in (*B*), within one minute the end of the chimney would swing over in that direction, Fig. 3, *D*. Within thirty minutes the chimneys would straighten and expand to their former size and shape.

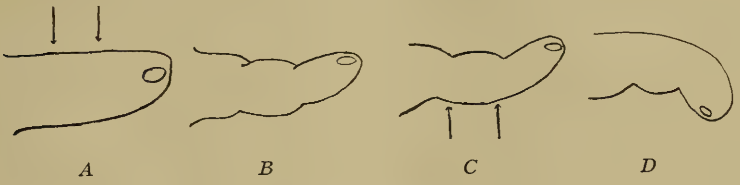

FIG. 3. The arrows indicate the points on the side of the chimney where the electrodes were applied.

STIMULATION AT BASE OF OSCULUM.

When a very weak stimulus was given for fifteen seconds with an electrode at each side of the base of the chimney, a very gradual longitudinal shrinking could be observed. The time required for the contraction to travel from the base to the tip was ten seconds, a rate of transmission of 0.17 mm. per second.

If the stimulus was a little stronger, an immediate contraction was followed by a slower general contraction, both lengthwise and in circumference, which would continue for about fifteen minutes, Fig. 4, *A* and *B*. The chimney would then begin to expand and within thirty-five minutes would be almost as large as before. On one occasion the sphincter at the base contracted more quickly than the others, resulting in the form of chimney shown in Fig. 4, *C*.

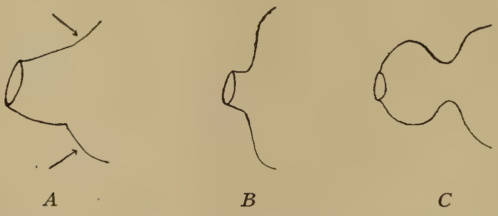

FIG. 4. The arrows indicate the points on each side of the base of the chimney where the electrodes were applied.

STIMULATION ON THE FLESH.

On several sponges the electrodes were applied immediately behind the osculum at distances of from 4 mm. to 2 mm. from it, and the flesh stimulated for periods varying from fifteen to sixty seconds. No effects could be seen on the chimneys.

There was, however, some indication of an excitation of the choanocytes. The currents were stronger and more rapid for from ten to fifteen minutes after the stimulation. Although this was taken as an indication of greater activity on the part of the flagellate cells, it is possible that the stimulation caused the ostia to dilate or open wider and in this way allow a larger volume of water to pass into the sponge body. It does not seem, however, that this would account for the increased rapidity with which the currents poured out of the oscula.

There was a noticeable difference in the responses of the oscula to Faradic stimulation depending on the strength of the electrical current used. From a weak stimulus,[1] a very gradual contraction was followed by a more rapid relaxation, while, if the stimulus was mild, there would be an immediate contraction followed by a slower more general contraction. In this case the chimney would remain contracted for a few minutes and then expand much more slowly than it had contracted. In the second instance, all of the contractile cells probably were directly stimulated by the electrical current, while in the first case it is probable that only those where the electrodes were applied were directly stimulated, the gradual contraction being the result of transmission of the stimulus from one cell to the next.

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[1] By a weak stimulus is meant one which could barely be detected when the electrodes were applied to the tongue, while by a mild stimulus is meant one that could be felt when the electrodes were applied to the lips.

In one instance, following a very weak stimulation for thirty seconds, it was possible to keep an accurate measurement of the time required for contraction and relaxation of the sphincter at the mouth of the osculum. No check was made on the general contraction or relaxation of the chimney. That the contraction was slower than the relaxation is shown by the following graph. The temperature of the water was about 27° C.

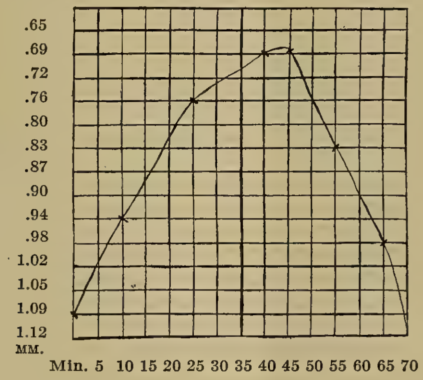

FIG. 5. Graph showing the rate of contraction and of relaxation of the sphincter at the mouth of an osculum following a weak Faradic stimulation. The abscissa represents the intervals of time in minutes and the ordinate the diameters of the opening in millimeters.

TRANSMISSION OF STIMULI.

It has already been pointed out that, apparently, very little transmission from one part of the body to another resulted from the application of the various types of stimuli used in these experiments. To determine if there was any transmission at all, a sponge which had two large oscula at a distance of about 5 mm. apart was selected. A strong stimulus was applied with the electrodes at the base of one chimney. The result was an immediate jerk of the entire chimney followed by a gradual and complete relaxation of the entire chimney from which it did not recover. The other chimney was not affected.

There is, however, a noticeable transmission of stimuli in the chimney itself and the rate of this transmission is more rapid from the tip to the base than from the base to the tip. This is shown in the experiments with electrical stimulation and by rubbing the chimney with a needle. Rubbing the edge of the mouth of the osculum with a needle started a downward wave of contraction which could be seen to travel at the rate of about 0.25 mm. per second. Rubbing at the base produced no effect. Weak Faradic stimulation at the tip caused a downward wave of contraction with a rate of about 0.35 mm. per second, while stimulation at the base produced a slight wave of contraction which traveled toward the tip at the rate of about 0.17 mm. per second.

Conclusions.

Ephydatia fluviatilis is a fresh-water sponge which lives in either quiet or flowing water. Swift currents cause a shortening of the oscular chimney but do not cause a constriction of the osculum. Placing the sponge in a small quantity of quiet water results in a cessation of activities and death of the sponge in a short time, probably because of the metabolic products which accumulate in the water and poison the sponge.

The edge of the mouth of the osculum seems to be more sensitive to stimulation that the remainder of the chimney, as shown by the effect of rubbing it in various places with a needle.

Cutting the body of the sponge or sticking a needle into it seemed to have no effect except the local effect on the tissues which were injured. That there is no transmission of the effects to surrounding tissues, even for a distance of 2 mm. or 3 mm. from the injury, is shown by the continued functioning of the parts as indicated by the currents coming from the oscula and by the fact that the chimney did not contract or in any way change its shape.

Subjecting the sponge to a low temperature does not have as disastrous an effect as increasing the temperature. Between 20° amd 30° C. seems to be approximately the temperature at which it thrives best. It is unable to live at a temperature of 40° C.

The oscular chimneys being continuous with the dermal membranes and themselves being contractile, one would expect to find the dermal membrane also contractile. It is capable of contraction to a slight extent under certain conditions. The chief contractile fibers, however, seem to be found only in the sphincters of the chimneys, which probably work against the general pressure of the water going out through the chimney.

Although in most cases, the responses of the fresh-water sponge were similar to the responses of *Stylotella*, there is one noticeable difference. This sponge responds to stimuli much more slowly, and the rate of transmission, where any at all is observable, is much slower.

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STUDIES IN MARINE ECOLOGY:

I. THE DISTRIBUTION OF COMMON LITTORAL INVERTEBRATES OF THE WOODS HOLE REGION.

W. C. ALLEE,

Marine Biological Laboratory and the University of Chicago.

The distribution of animals within the Woods Hole region has been well studied by men interested in individual species and by those concerned with general faunistic problems. Some aspects of the ecology of the region are thoroughly set forth by Verrill and Smith in their "Report on the Invertebrate Animals of Vineyard Sound" made fifty years ago. This classic study remains the best account of the ecology of littoral species available.

The extensive "Biological Survey of the Waters of Woods Hole and Vicinity"[1] completed about ten years ago by Sumner, Osburn and Cole, while a mine of information concerning the animals of the region, was directly concerned with dredging operations and has little to say at first hand concerning the animals of the intertidal region or those found just below the tidal zone. Among other suggestions they recommend (p. 25) that the intertidal fauna should receive the same detailed attention that they have given to the bottom dwelling species.

In the absence of a report by the person best qualified to write on the subject, Mr. George M. Gray, the present series of papers has been prepared to make available information accumulated in nine consecutive summers' experience with the inshore invertebrates of the region.

The work has been done in connection with a teaching appointment in the course of Invertebrate Zoölogy of the Marine Biological Laboratory. It represents the collaboration of eighteen staff members and of about four hundred students. Many of the present collecting methods were installed in conjunction with Professor Caswell Grave, my predecessor in charge of the course, but the records here used have been kept from the beginning by myself with the occasional help of other staff members.[1]

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[1] This report will be referred to hereafter as the *Biological Survey*.

The records are based on the bi-weekly collecting trips of the Invertebrate Class and cover most thoroughly the period from about June 20 to August 15. These trips have been supplemented by expeditions made by instructors and by special trips for particular observations.

The organization of field work for eight years has been to divide the class into as many collecting teams as there were instructors. One person from each team was appointed recorder for the day and was supplied with a list of all the animals previously taken from the locality under consideration. The animals found were recorded according to habitats. The complete list for the year was made up from these combined records.[2]

[1] The following people have been at one time or another members of the instructing staff of the Invertebrate Course and have contributed to the data on which this series of papers is based. Without their coöperation this work could not have been done. Caswell Grave, Raymond Binford, E. J. Lund, George A. Baitsell, T. S. Painter, F. M. Root, W. J. Kostir, Robert H. Bowen, C. L. Parmenter, G. S. Dodds, Robert Chambers, Jr., Ann H. Morgan, W. J. Crozier, Donnell B. Young, J. P. Visscher, J. A. Dawson, Christianna Smith and E. A. Adolph.

I am indebted also to Mr. G. M. Gray for much valuable aid and friendly assistance; to Dr. Mary J. Rathbun for identification of the Brachyura; to Mr. Waldo L. Schmidt for similar service with the Anomura and Macrura; to Mr. Clarence R. Shoemaker for similar service with the amphipods and isopods; to Professor E. S. Morse for assistance with some of the molluscs and to Professor Raymond Osburn for assistance with the Bryozoa.

[2] The formal record of collecting experience has been recorded in abbreviated form on library cards which are deposited in the Library of the Marine Biological Laboratory. An annotated catalog of the distribution has been prepared as Study II. of this series and deposited with the library of the U. S. Fish Commission who have kindly agreed to furnish copies to the libraries of the Marine Biological Laboratory at Woods Hole; the Museum of Comparative Zoölogy at Cambridge, Scripps Institution at LaJolla; the United States National Museum at Washington and to the Harpswell Laboratory at Mount Desert Island, Maine.

The catalog shows the littoral invertebrates collected during the years 1915–1921 inclusive. Each locality in which an animal has been taken is recorded. The number of years which it has been found in a given locality is shown and an index figure of comparative abundance is also given. Where possible and desirable the location of particularly favorable collecting grounds is given with some exactness. This elaborated catalog forms the basis from which the facts presented here are drawn and together with the present report gives the background for the two following studies.

So far as possible, identification was done in the field. Doubtful specimens were referred from one instructor to another. Specimens new to the locality or difficult to identify were brought into the laboratory for further study. With the exception of the arthropods, few of the specimens have been referred to experts although we have gradually accumulated a type collection of animals found. The identification of animals has been made on the conservative basis that when doubt existed, the specimen was referred to the more common species. Wild identifications have been eliminated as far as possible, even to the extent of throwing out the entire reports of inexperienced instructors.

In spite of this care, mistaken identifications have probably been turned in and accepted. The list here given is substantially correct since the animals have either been reported by qualified collectors or placed on the list from demonstrated specimens. The imperfections lie largely in failing to distinguish closely related species and in possible errors in distribution records.

II.

The collections upon which this series of reports are based have been made largely in the littoral zone as defined by Edward Forbes; that is, between high water and a depth of two fathoms. This is not the littoral zone of modern zoölogists, but the term has been used with so great a variety of meanings that the extent of the study can be more easily and definitely located as being in the intertidal and ad- or sub-tidal regions.[1]

The intertidal zone is much restricted in the Woods Hole region on account of the slight rise and fall of the tides. The tide tables of the U. S. Bureau of Commerce show a spring tide range of about five feet for this section of Buzzards Bay and only about two feet for Vineyard Sound stations.

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[1] Murray and Hjort use the term "littoral zone" to include the region near the shore down to a depth of 30 or 40 meters: "almost as far as there are sea-weeds." It is frequently used as by Petersen to include the entire continental shelf. The botanists tend to be more exact. Kjellman limits the term to the region between extreme low and extreme high tide. Davis regards the littoral zone as extending from about mean low water to the highest point at which algæ can grow. Flattely and Walton ('22) follow Cotton ('12) and define the littoral region as extending from the level of highest marine vegetation, to low water at neap tide.

I prefer to use littoral in its original meaning of "pertaining to the shore"; intertidal or tidal zone adequately and exactly describes the region between the tide lines, and sub-tidal or adtidal are exact terms, if not the most correct etymologically, for the region below low tide. The question is discussed in the *Biological Survey*, p. 179.

Studies have been made in the following localities:[1]

WHARF PILINGS.

Crane's Wharf Pilings.—This is a comparatively new wharf located near the public steamboat wharf in Great Harbor at Woods Hole. At the shore end the water at low tide comes close to the retaining wall and some collecting has been done annually in the crevices of the wall. The water at the outer end is over twelve feet deep. The number of species and of animals on these pilings has increased noticeably during the period of observation.

Vineyard Haven Wharf Pilings.—The old New York and Portland Wharf on the south side of Vineyard Haven is located well out toward the Sound. The water here comes up on a sandy beach which at low tide is bare for a considerable distance under the wharf. At the outer end the Government Chart shows 11 feet of water. In my experience the water is deeper. This is an old wharf with many pilings rotted off below water level. Some of the pilings are reproduced in the American Museum of Natural History in New York. Collections from both wharfs were made from boats by means of the usual scrape nets.

Marine Biological Laboratory Pier on Glass Slides.—For a number of years, glass slides have been placed under the M. B. L. pier in connection with other studies. In 1921 the slides were carefully examined by Dr. D. B. Young and myself after they had been suspended in water under the pier from July 1 to August 9 at a depth of about six feet. The M. B. L. supply float containing animals from all parts of the region was only a few feet away and accordingly more species were attached than might normally be found.

ROCKS AND FLATS.

Hadley Harbor, Southwest and Southeast Gutters.—These naturally narrow rocky gutters have been further narrowed artificially

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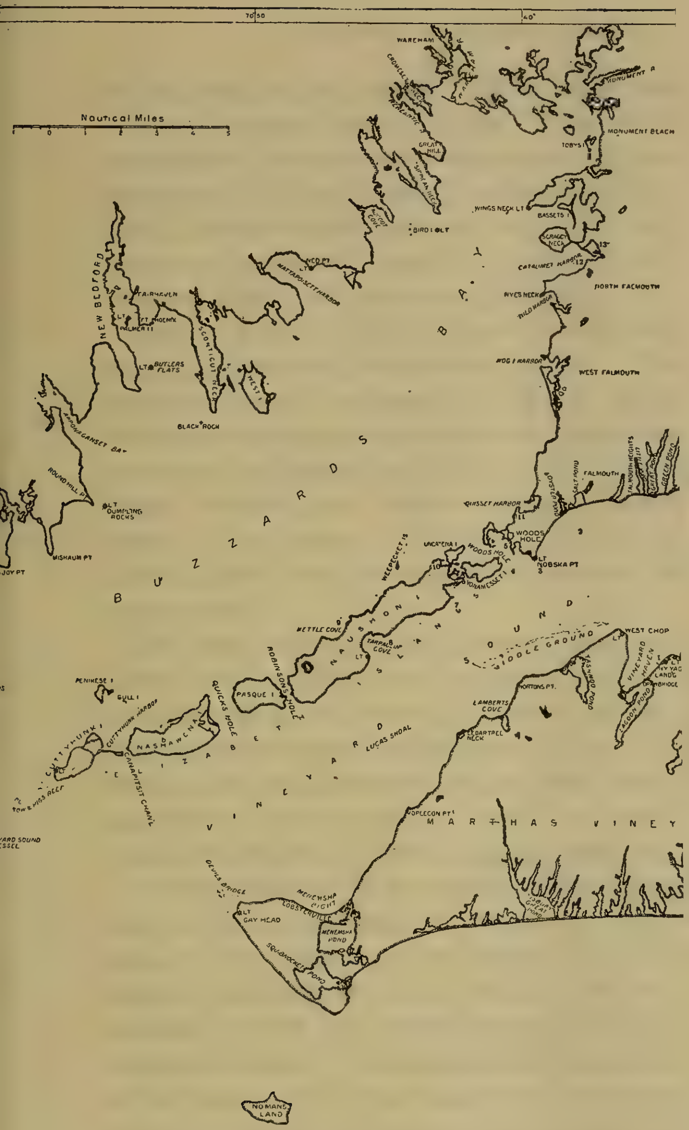
[1] Consult map.


70°50
40'
Nautical Miles
0 1 2 3 4 5
WAREHAM
MONUMENT
MONUMENT BEACH
TOBYS I
GREAT HILL
WINGS NECK LT
BASSETS I
BIRD I LT
SCRAGGY NECK
CATAUMET HARBOR
NORTH FALMOUTH
NYES NECK
WILD HARBOR
HOG I HARBOR
WEST FALMOUTH
NED PT
MATTAPOISETT HARBOR
NEW BEDFORD
FAIRHAVEN
FT PHOENIX
PALMER I
BUTLERS FLATS
SCONTICUT NECK
WEST I
BLACK ROCK
APPONAGANSET BAY
ROUND HILL PT
DUMPLING ROCKS
MISHAUM PT
JOY PT
BUZZARDS BAY
FALMOUTH
FALMOUTH HEIGHTS
OYSTER POND
SALT POND
GREEN POND
QUISSET HARBOR
WOODS HOLE
NOBSKA PT
UNCATENA I
WEEPECKET IS
NAUSHON I
KETTLE COVE
TARPAULIN COVE
PASQUE I
ROBINSONS HOLE
QUICKS HOLE
NASHAWENA I
PENIKESE I
GULL I
CUTTYHUNK I
CUTTYHUNK HARBOR
CANAPITSIT CHANL
VINEYARD SOUND
LUCAS SHOAL
MIDDLE GROUND
WEST CHOP
VINEYARD HAVEN
TASHMOO POND
LAGOON POND
NORTONS PT
LAMBERTS COVE
CEDAR TREE NECK
MARTHAS VINEYARD
DEVILS BRIDGE
MENEMSHA BIGHT
LOBSTERVILLE
GAY HEAD
MENEMSHA POND
SQUIBNOCKET POND
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yard Haven Wharf.
on 1, Vineyard Sound; near oyster beds.
on 2, Vineyard Sound; *Chætopleura* grounds.
on 3, Vineyard Sound; *Amarœcium pellucidum* grounds.
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e's Wharf. Localities 2 and 3 in Harbor dredging are nearby.
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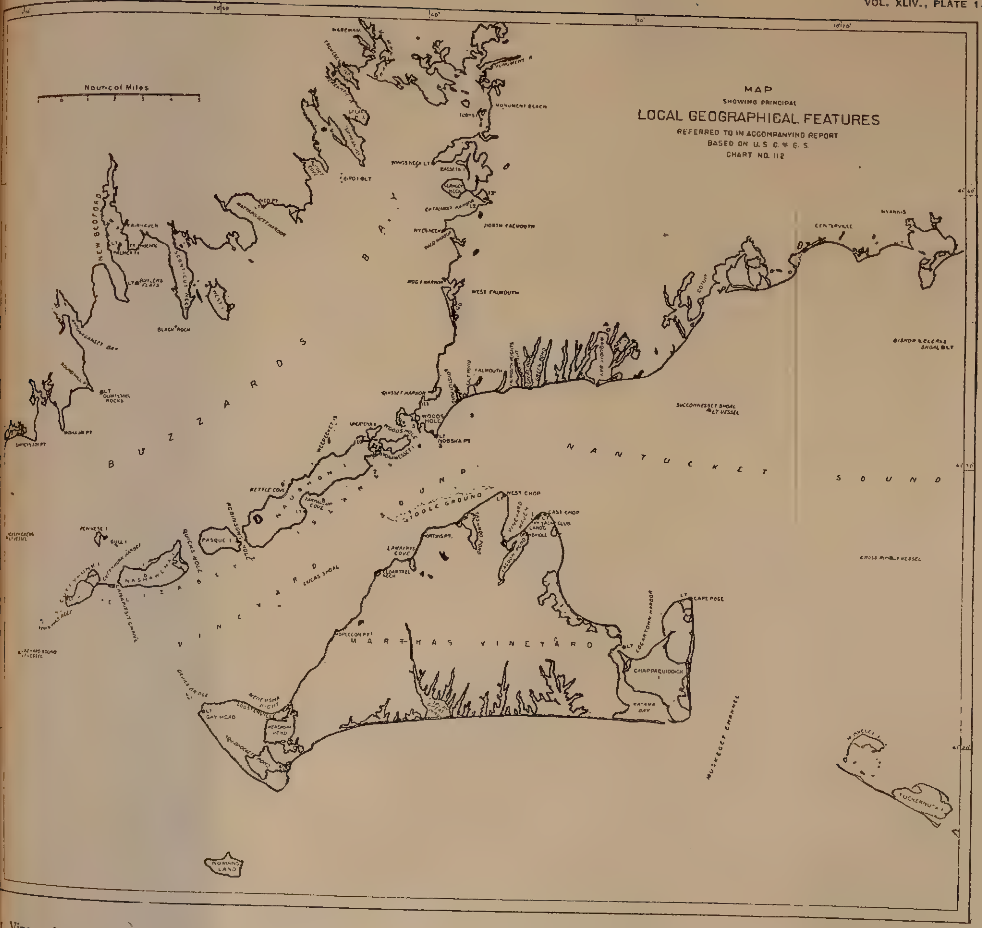
MAP
SHOWING PRINCIPAL
LOCAL GEOGRAPHICAL FEATURES
REFERRED TO IN ACCOMPANYING REPORT
BASED ON U. S. C. & G. S.
CHART NO. 112
Nautical Miles
WAREHAM
MONUMENT BEACH
WINGS NECK LT
BASSETS I
BIRD I LT
CATAUMET HARBOR
NORTH FALMOUTH
NYES NECK
WEST FALMOUTH
FALMOUTH
QUISSET HARBOR
WOODS HOLE
NOBSKA PT
NEW BEDFORD
FAIRHAVEN
BLACK ROCK
B U Z Z A R D S   B A Y
V I N E Y A R D   S O U N D
N A N T U C K E T   S O U N D
MIDDLE GROUND
KETTLE COVE
PASQUE I
PENIKESE I
NASHAWENA
QUICKS HOLE
ROBINSONS HOLE
LUCAS SHOAL
WEST CHOP
EAST CHOP
LAMBERTS COVE
M A R T H A S   V I N E Y A R D
CHAPPAQUIDDICK
CAPE POGE
KATAMA BAY
MUSKEGET CHANNEL
GAY HEAD
NOMANS LAND
CENTERVILLE
HYANNIS
SUCCONNESSET SHOAL LT VESSEL
BISHOP & CLERKS SHOAL LT
CROSS RIP LT VESSEL
TUCKERNUCK I
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1. Vineyard Haven Wharf.
2. Station 1, Vineyard Sound; near oyster beds.
3. Station 2, Vineyard Sound; *Chætopleura* grounds.
4. Station 3, Vineyard Sound; *Amarœcium pellucidum* grounds. Locality 1 of the dredging in Great Harbor is just within the harbor entrance.
5. Crane's Wharf. Localities 2 and 3 in Harbor dredging are nearby.
6. Lackey's Bay and Sound Gutters. The figure stands approximately on Blind Gutter Bar.
7. Station 4, Vineyard Sound; starfish hole.
8. Station 5, Vineyard Sound; sand dollar bed.
9. Kettle Cove.
10. Northwest Gutter Flats.
11. Gansett Bay.
12. North Falmouth Flats.
13. Squeteague Harbor in the North Falmouth Flats complex.

The map is taken from Bull. 31, U. S. Bureau of Fisheries.

so that they can be bridged. Strong tidal currents run through them the greater part of the day. They are relatively shallow, rock-walled channels, containing about six feet of water and are connected with open water by creeks which are also rock edged. Small patches of mud and sand occur frequently and the whole system of protected waterways supports many plants, *Ascophyllum, Fucus* and *Sargassum filipendula.*

Hadley Harbor Flats, Northwest Gutter.—Northwest Gutter separates Uncatena Island from Naushon. Before opening into Buzzards Bay it enlarges to form an approximately square expanse of shallow water about 250 yards along the south and west sides and about 400 yards in greatest diagonal. In most places the water is so shallow that it is difficult to push a boat along at low water. The sand bar over the channel of the gutter is fully bare at extreme low tide often to the extent of an acre or more.

The wide channel is kept scoured clean by the current, but behind the protecting sand and gravel spits, organic debris has accumulated to the depth of several feet and supports a rank plant growth composed chiefly of eel grass. At the Bay entrance there is the usual accumulation of rocks which extend off to a sand bottom some four feet below the lowest tide. The mud flats are bordered by rocks partially buried by mud.

Gansett.—Gansett is an offshoot of Quamquissett Harbor and has the same opening into Buzzards Bay. The main axis extends at an angle from the opening so that the back portion is usually protected from the direct drive of the waves. The opening is about 200 yards wide and the bay is approximately twice that length. At mid-mouth at mean low tide the water is 18 feet deep. The sides slope in rapidly near the shore so that there are only narrow strips of the different habitat zones. At the sides are the customary rocks and the outer corners are guarded by rock piles. At the head of the bay the shore is sand mixed first with gravel and lower with mud. Eel grass comes within two rods of the water's edge at low tide and thickly covers the bottom throughout its extent.

North Falmouth.—The collecting grounds here are scattered. They are located at the head of Cataumet Harbor and extend over Squeteague Harbor which opens from the former by a winding narrow passage. Except for the dredged passage, most of the region can be waded at low tide. Much of the ground in Cataumet and almost all in Squeteague Harbor is left bare by the spring tides. The collecting is over a wide range of bottom: sand, mud, scattered rocks and gravel with and without eel grass and other sea-weeds. There is a somewhat sparse collection of rocks along the shore line.

Lackey's Bay.—Lackey's Bay belongs to the Hadley Harbor complex. It is located on the Vineyard Sound side between Naushon and Nonamasset. The part studied forms an expanded entrance to Middle Gutter which, by the construction of a causeway, has become Blind Gutter. The current is much diminished by the causeway and the inner part of the bay is deeply overlaid with muck. Eel grass is abundant. The region most studied is about 400 yards long by 200 yards wide and is separated from the Sound by a sand bar which is left bare at low tide.

DREDGING.

The dredging has been largely in three localities in Vineyard Sound. These are the sand dollar bed (Map, No. 8) near the east side of the entrance to Tarpaulin Cove in about 20–30 feet of water. The bottom material brought up by the coarse dredge used is largely composed of shells. The starfish hole (Map, No. 7) is further east and still off Naushon, has about 90 feet of water. The *Chætopleura* grounds (Map, No. 3) off Nobska have about sixty feet of water. The bottom is decidedly pebbly. Some dredging has been done further east on or near the planted oyster bed (Map, No. 2) in Falmouth Harbor. The bottom here is sand and gravel in about 60 feet of water. In 1921 we dredged off the west entrance from Vineyard Sound to Great Harbor (Map, No. 4) in about 80 feet of water. This is over an *Amarœcium pellucidum* bed.

In 1920 we dredged in Great Harbor (Map, No. 5): at the east end of Nonamasset in 10–12 feet of water; in the Fish Commission Hole at a depth of 50 feet and at the West end of the passage in Woods Hole in about 20 feet of water.

The dredging work has been largely incidental and the results are given chiefly as a means of comparing the more extensive results obtained by the dredgings of the *Biological Survey* with our main work further inshore.

A Habitat Check List of the Common Invertebrate Animals of the Woods Hole Littoral with Distribution Records for 1920 and 1921.

The appended list of animals is based on all the collecting done since 1912. The distribution records are based on the reports from operations in 1920 and 1921. The statistics given are from team records. Thus in these two years, two teams collected *Chalina* from the mud, eight teams have recorded it from rocks, ten from wharf pilings and nine from dredging. The figures given show no indication of the number of specimens taken other than that suggested by the fact that the more animals present, the greater the probability that all teams would find them. Anyone interested in the abundance of these animals in particular localities is referred to the second study in the present series.

The tabulation is from the reports of 30 collecting teams operating on wharf pilings; 52 from sand, mud, gravel and eel grass; 56 teams from rocks and 42 from dredging. The records of plankton have been kept in a different way and the presence of recognized animals in late July or early August is merely checked.

The classification of habitats in the field has sometimes been left to the judgment of the student recorder and it is entirely probable that some of the 52 recorders thought a given habitat was best recorded as "sand" while others regarded animals from a similar place as "mud" dwelling. All gradations between the two exist and the conditions under which the collecting was done do not permit a more refined grading. The error arising from this source is somewhat compensated by the fact that no dragnet collections were made as it was desired to find where individual animals live as well as to collect different species.

Unidentified animals have not been included in the habitat list unless the genus, at least, could be determined with some assurance. All the records are for living animals since for ecological purposes the recording of dead shells can only be worthless and confusing in a region where tidal currents run strongly and where the shore birds distribute shells even over the land.

The nomenclature follows Pratt wherever the species are listed in his *Manual*. Other species have the name given them in the catalog of the *Biological Survey*. No attempt has been made to give synonyms since these can usually be found in the *Survey* catalog.

The arrangement of species within the major divisions is alphabetical. While this does violence to all principles of taxonomy, the taxonomic sense is not strongly developed at present, and this method renders the material more easily available to the average zoölogist than would be the case if a strictly taxonomic system were followed.

The records given in the habitat list are necessarily abbreviated. Thus, *Hydractinia* is recorded as taken from "sand" when the whole record should read: "on shells inhabited by hermit crabs taken on sandy bottom"; or other animals, as *Sagartia luciæ*, recorded from "mud" which does not mean that the anemone was growing on the mud but that it was found attached to a bit of board or rock surrounded by typical mud conditions.

The classification headed "eel grass" includes records of animals living free among the eel grass, as *Pecten;* attached to eel grass, as *Pennaria;* crawling over it, as *Ophioderma;* on the substratum at its base, as *Microcione;* or burrowing in the substratum at its roots, as *Cumingia*. In addition the lumping is still greater for one must remember that eel grass begins to grow on fairly pure sand and extends back to the pure muck of the flats.

A number of animals are recorded under "rocks and rockweeds" which were taken only from the substratum under or among the rocks. Whenever all the records are for an animal so found, the entry has been appropriately labeled.

Under "range" is listed the information at hand showing the distribution of the animal along the Atlantic Coast. The abbreviations are: N., north ranging; S., south ranging; M., approximately mid-range; L., local; C., cosmopolitan. Whenever an animal is known to extend twice as far north of Woods Hole as south, it is listed as north ranging and *vice versa*. (Cf. Hoyle, 1889). Some relations between the local and geographical distribution will be discussed in a later paper.

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
			No. Teams Reporting.							
			52	52	52	52	56	30	42	
PROTOZOA. List not given.										
PORIFERA.										
Chalina arbuscula	S.		2				8	10	9	
Cliona celata (?)	S.			2		1	33	25	16	
Grantia ciliata	N.			1			11	30	13	
Leucosolenia botryoides	N.						13	24	1	
Microciona prolifera	S.		1			4	43	30		
Tethya gravida	L.		1						1	
CŒLENTERATA. *Hydrozoa*										
Abietinaria abietina	N.								12	
Bougainvillia carolinensis	S.					1	2	1		✓ sp.?
Campanularia calceolifera								1		
Campanularia sp.?							1	5		
Clava leptostyla	N.						1			
Clytia grayi	L.						2			
Clytia bicophora	N.					1	1	1		✓ sp.?
Clytia cylindrica						1				
Eucheilota sp.? *medusæ*										✓
Eudendrium album						2	4	6		
Endendrium ramosum	M.						7	22	7	
Eudendrium tenue								1		
Gemmaria gemmosa	S.			1			1	1		✓
Gonionemus murbachii	L.									
Hydractinia echinata	N.		9	18	3	5	12		11	
Obelia bicuspidata	S.					1		1		
Obelia commissuralis	S.		1			12	8	11	2	✓ sp.?
Obelia geniculata	N.					5	6	7		
Pennaria tiarella	S.					14	4	10	4	✓
Phialidium sp.? *medusæ*										✓
Podocoryne carnea	N.			2		1				
Schizotricha tenella	S.						1	11	2	
Sertularia pumila	N.					11	30	12	10	✓
Stylactis hooperi	S.									
Tubularia crocea	S.							30	7	✓
Scyphozoa.										
Aurelia flavidula	M.									
Cyanea capillata arctica	N.									
Dactylometra quinquecirrha	S.									
Haliclystus auricula	N.									

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
			No. Teams Reporting.							
			52	52	52	52	56	30	42	
			Anthozoa.							
Astrangia danæ	S.						11		7	
Alcyonium carneum	N.									
Edwardsia elegans	N.		1	5		1				
Eloactis producta	S.			3						
Metridium dianthus	N.						29	24		
Sagartia leucolena	S.		3	3		2	37	11	4	
Sargartia luciæ	S.		2	2		6	38	6	2	
Sagartia modesta	S.					4	12 under			
			PLATYHELMINTHES.							
			Turbellaria.							
Bdelloura candida	S.	3	5	2		7				
Bdelloura propinqua		1								
Polychœrus caudatus	N.		1	3		5	2	1	1	
Procerodes wheatlandi	N.						1			
Stylochus ellipticus	N.		3	2	1	1	6	3		
Stylochus zebra	L.						1		1	
Syncœlidium pellucidum						2				
			Nemertini.							
Amphiporus ochraceus	S.								5	
Cerebratulus lacteus	S.		7	11		2	2 under			✓
Cephalothrix linearis	N.						1 under			
Lineus sp.?			2	6		5	1		2	
Lineus bicolor	S.		1	1			1	2		
Micrura leidyi	M.		6	15		8	3 under			
Tetrastemma vermiculum	S.							5	3	
			NEMATHELMINTHES.							
Pontonema marinum	S.		1	1		5	6	19	6	
			ECHINODERMA.							
			Asteroidea.							
Asterias forbesi	S.		1	8		4	28	14	29	✓
Asterias vulgaris	N.							1	4	
Henricia sanguinolenta	N.						6	4	11	

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
			No. Teams Reporting.							
			52	52	52	52	56	30	42	
Ophiuroidea.										
Amphipholus squamata	N.						2		12	
Ophioderma brevispina	S.					5				
Echinoidea.										
Arbacia punctulata	S.			6			18	2	17	✓
Echinarachnius parma	N.						1		12	
Strongylocentrotus drœbachiensis	N.									
Holothuroidea.										
Leptosynapta inhærens	S.		11	24		10				
Thyone briareus	S.		25			6			2	
ANNELIDA.										
Archiannelida.										
Dinophilus sp.?										✓
Polychæta										
Ampharete setosa	S.									
Amphitrite attenuata							1	9		
Amphitrite brunnea	N.							1		
Amphitrite ornata	S.		19	8		3	3 under		2	
Arabella opalina	S.		8	17	2	5	1 under	1	7	
Arenicola cristata	S.		3	6			1 under			
Autolytus cornutus	N.					1		9		
Autolytus varians	S.							1		
Chætopterus pergamentaceus	S.					1				
Cirratulus grandis	S.		11	6	1		5 under		1	
Cirratulus tenuis									1	
Capitella sp.?										
Clymenella torquata	M.		17	25		6	6 under			
Diopatra cuprea	S.		6	14		2	1 under		2	
Drilonereis longa			1	7	1					
Enoplobranchus sanguineus	M.		3							
Glycera americana or *dibranchiata*	S. M.	}	20	53	1	12	1 under			
Harmothœ imbricata	N.		2	5	1	3	26	27	11	

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
			No. Teams Reporting.							
			52	52	52	52	56	30	42	
			Polychæta Con't.							
Hydroides hexagonus	S.	5	2	3	1	3	39	17	22	
Laonice viridis	S.	3	3	1						
Lepidametria commensalis	S.						1 among			
Lepidonotus squamatus	N.,	1	3	5	2	2	37	29	22	
Lepræa rubra	S.				1		4 under			
Lumbrinereis hebes										
Lumbrinereis tenuis	S.		7	19	2	1	3 under		14	
Maldane urceolata	S.		3	9		2				
Marphysa leidyi			1						3	
Nereis limbata	S.		12	9	2	3	8	3		√sp.?
Nereis pelagica	N.		4	6			9	29	22	
Nereis virens	N.		17	20		9	3 under	1		
Nicolea simplex			1	3		2	7	13	7	
Pectinaria gouldi	S.		9	18	1	5				
Potamilla sp.?									1	
Pholoë sp.?										
Phyllodoce catenula	N.	1	3	6		3	5	17	13	
Pista palmata	S.		3	1		1				
Platynereis megalops	S.					1		1		
Podarka obscura	S.		2			4	4	7	6	
Polycirrus eximeus	S.		10	14		4	15	6	12	
Polydora sp.								2		
Sabella microphthalmia	S.		1	1	2		3	7	1	
Sabellaria vulgaris	S.	1	2	2		5	6	2	4	
Scoloplos acutus			12	1		6				
Scoloplos fragilis	S.		8	19		9	1 under			
Scoloplos robustus	S.		8	10		3	1 under			
Spio (setosa?)				1						
Spirorbis spirorbis	N.		3	4	2	11	26	19		
Spirorbis tubæformis	S.						1		1	
Sthenelais leidyi	S.		6	5		4	3	4	6	
Terebellides strœmi	N.									
Thelepus cincinnatus	N.						1	3		
Trophonia affinis	S.		4	1					6	
			Chætognatha and Sipunculoidea							
Phascolosoma gouldii	M.		13	22		3	2 under			
Sagitta sp.?										√

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
			No. Teams Reporting.							
			52	52	52	52	56	30	42	
BRYOZOA.										
Ætea anguina	N.					5	10	12		
Alcyonidium sp.?							3			
Bicellaria ciliata	M.							3	3	
Bowerbankia gracilis	N.						3	1		
Bugula cucullifera	N.							1		
Bugula flabellata	N.						1	1		
Bugula turrita	S.		1	2		17	17	31	14	✓
Cribrillina punctata	N.								1	
Crisia eburnea	N.		1	1		4	21	18	12	
Flustrella hispida	N.					1	22			
Hippothoa divaricata	C.							1		
Hippothoa hyalina	C.							1		
Lepralia pallasiana	N.									
Lepralia pertusa	S.	1		1		10	14	6	6	
Lepralia serrata	M.							1		
Lichenopora verrucaria	N.							2		
Membranipora monostachys								1		
Membranipora pilosa	N.			1	1	6	21	13	5	
Membranipora tenuis									6	
Microporella ciliata	C.						1	2	3	
Schizoporella biaperta									5	
Schizoporella unicornis	S.		2	3	1	8	32	25	11	
Smittia trispinosa nitida	M.					1	3		15	
ARTHROPODA.										
Phyllopoda.										
Evadne nordmanni	M.									✓
Podon leuckarti	M.									✓
Cirripedia.										
Balanus balanoides	M.		5	4	3	2	48		2	} ✓
Balanus eburneus	S.		1	3	1	6	18	19	17	
Lepas anatifera	C.	1					1			
Arthrostraca.										
Amphithœ rubricata	N.		1	1			6	6	8	
Autonœ (Lembos) smithi	L.			1			5	5	9	
Æginella longicornis	N.						1	4		
**Caprella geometrica*	S.			1		6	13	26	1	✓
Chiridotea cœca	S.		2	2		2	2			
Corophium cylindricum								2		
Cyathura carinata	N.			1		1				
Edotea triloba	M.	1	3			2	2			

* In part *Æginella*

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
			No. Teams Reporting.							
			52	52	52	52	56	30	42	
Arthrostraca Con't.										
Erichsonella filiformis	N.			1		2	2	2	4	
Gammarus (Sev. sp.)		2	8	11	3	15	10	28	18	
Haustorius arenarius	S.			2						
Idothea baltica	M.		5	4		20	9	15		
Idothea metallica	S.		3		2	5				
Idothea phosphorea	N.						1			
Jæra marina	N.	2		2	1	4	3			
Ligyda oceanica	N.						3			
Orchestia agilis	S.	10	1	33			8	2		
Sphæroma quadridentatum	S.						5			
Talorchestia longicornis	S.		2	14	6	1				
Tanais cavolinii	N.							1		
Unicola irrorata	N.								1	
Thoracostraca.										
Callianassa stimpsoni	S.		1				1 among			
Callinectes sapidus	S.		1	1						
Cancer borealis	N.					3				
Cancer irroratus	M.		5	7	2	4	6	2 near	2	
Crangon vulgaris (*Crago septemspinosus*)	M.		3	4	4	16	3		1	
Carcinides mænas	S.		16	4	4	4	8		5	
Heterocrypta granulata	S.								1	
Heteromysis formosa	N.	1	3	3		1	1		12	✓
Hippa (*Emerita*) *talpoida*	S.									
Hyas coarctatus	N.									
Libinia dubia	S.		14	8	1	10	3	20	16	
Libinia emarginata	S.		14	4	1	7	1	10	9	
Lysiosquilla armata										
Mysis stenolepis	M.									
Michtheimysis mixta										
Ovalipes ocellatus	S.		1	3		3	2		1	
Pagurus acadianus	M.			1					2	
Pagurus longicarpus	S.	3	19	16	5	10	7	2	22	
Pagurus pollicaris	S.	1	4	10	4	7	1	1	10	
Palæmonetes vulgaris	S.		9	7		16	8			
Panopeus sayi or *Neopanope texana sayi*	S.	2	17	12	3	12	7	27	16	
Pelia mutica	S.			1						
Pinnixa chætopterana			2			2	1 among			
Pinnixa sayana (*cylindrica*)										
Pinnotheres maculatus	S.		3					6		
Portunus sayi	S.									
Squilla empusa	S.									

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
			No. Teams Reporting.							
			52	52	52	52	56	30	42	
Thoracostraca Con't.										
Uca pugilator	S.		21	4	3	2	8			
Uca pugnax	S.			2						
Virbius (Hippolyte) zostericola	S.	1	4	2		25	1			
Arachnoidea.										
Anoplodactylus lentus	N.	2					4	10	2	
Pallene empusa	S.					2	1	5		✓
Limulus polyphemus	S.	1	12	10	2	6	1			
Tanystylum orbiculare	S.							10	1	
MOLLUSCA. *Amphineura.*										
Chætopleura apiculata	S.	3					19		16	
Gasteropoda.										
Acmæa testudinalis	N.	2	1	2		1	26	1	3	
Bittium alternatum	M.	1	6	6	2	20	17	11	8	
Busycon canaliculatum	S.			7	1		2			
Busycon carica	S.			1	1		1	1		
Cæcum pulchellum							2			
Cerithiopsis emersonii	S.	1				2	3		6	
Cerithiopsis greenii	S.					2	1 sp.?		2	
Cerithiopsis terebralis									2	
Columbella avara	S.	1	3	5	1	9	13	14	19	
Columbella lunata	S.	1	3	7		12	20	30	21	
Coryphella gymnota	N.	1			1	1	2	15		
Crepidula convexa	S.	4	12	10	1	6	20	4	7	
Crepidula fornicata	S.	2	6	15	1	2	21	12	9	
Crepidula hlana	S.	3	7	16	2	2	15	14	15	
Doris bifida	N.							1	1	
Elysia chlorotica	M.					4	1			
Eupleura caudata	S.		1	3	1		4		5	
Lacuna vincta	S.		4	4	2	14	13	21	9	
Littorina irrorata	S.						2			
Littorina litorea	N.	1	15	8	6	18	29	27		
Littorina palliata	N.		4	5	3	11	24	7	1	
Littorina rudis	N.		8	4	2	12	32	6	1	
Melampus lineatus	S.		14	4	2					
Nassa obsoleta	S.		35	14		3	9			
Nassa trivittata	S.		13	16	1	3	8	11	3	
Nassa vibex	S.		1							
Natica duplicata	S.		2	6	1	3	1 among			
Natica heros	M.		2	1	1		2			

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
			No. Teams Reporting.							
			52	52	52	52	56	30	42	
Gasteropoda Con't.										
Natica immaculata	N.								2	
Natica pusilla	S.		1			1				
Odostomia sp.?		1	2	1		7	24	2		
Rissoa minuta	N.		3							
Rissoa sp.?										
Scalaria sp.?			1	1						
Purpura lapillus	N.			1			10			
Urosalpinx cinerews	S.	4	5	8	1	5	31	22	5	
Pelecypoda.										
Anomia aculeata	N.					1	12	4	5	
Anomia ephippium	S.		2			1	22	10	7	
Arca pexata	S.			1			5	1	7	
Arca ponderosa	S.									
Arca transversa	S.		1	1			1		4	
Astarte castanea	M.								3	
Astarte undata	N.								5	
Cardium pinnulatum	N.								5	
Clidiophora trilineata	S.								3	
Corbula contracta	S.			2						
Cumingia tellinoides	S.		10	11		6	1			✓
Ensis directus	M.		9	9		2	5 among			
Gastranella tumida	S.							1		
Gemma gemma	N.			3			1			
Lævicardium mortoni	S.		11	5	2	4	1 among			
Lyonsia hyalina	S.	1		2						
Macoma tenta	S.		2	2		1			1	
Mactra lateralis	S.			1						
Mactra solidissima	N.			3			2		2	
Modiolus demissus	S.		22	15	3	6	8			
Modiolus modiolus	N.		10	7		4	6	3	3	
Mya arenaria	N.		23	21	3	6	5 among			
Mytilus edulis	N.		11	11	5	4	24	28	7	
Nucula delphinodonta	N.								1	
Nucula proxima	M.		3	3	1	4	4 among		3	
Ostrea virginica	S.		10	5			9			
Pecten irradians	S.		9	9		11	4		4	
Petricola pholadiformis	S.		3	2	3		1			
Saxicava arctica	N.						2			
Solemya velum	M.		10	20		4	4 among			
Tellina tenera	S.		5	16	3	4	1			
Teredo navalis	N.	19		2	1	1	1	7		

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Derdging.	Plankton.
			No. Teams Reporting.							
			52	52	52	52	56	30	42	
Pelecypoda Con't.										
Venus mercenaria	S.	1	19	14	2	1	4 among		2	
Yoldia limatula	N.									
Zirphæa crispata	N.		1							
Cephalopoda.										
Loligo pealii	S.			3						✓
CHORDATA.										
Appendicularia longicauda	M.									✓
Amarœcium constellatum	N.	3			2	3	19	29	14	
Amarœcium pellucidum	S.								12	
Amarœcium stellatum	S.									
Botryllus schlosseri	N.	1				13	7	5		✓
Didemnum lutarium	N.	2				6	13		30	
Dolichoglossus kowalevskyi	S.		12	14	6	2	3 under			
Molgula manhattensis	S.	2	3		1	6	8	24	1	
Molgula papillosa	N.							1		
Molgula arenata	S.							1		
Perophora viridis	S.	2		1		5	16	28	4	
Styela partita	S.		1	1		6	17	29	9	

IV.

The entire mass of data available was analyzed in 1917 and again in 1920 in an attempt to discover the relationships existing between animal associations from the different types of environments. The first type of analysis was planned to discover the number of species common to different combinations of these habitats and conversely to find the number of animals peculiar to each type of habitat. The analysis was not repeated after the 1921 records were available because it was not considered that these records would materially change conclusions already arrived at by the preceding work.

The analysis of the records from 1915–1920 inclusive follows:

Habitats.	Number of Species.
Flats, rocks, pilings and dredgings	54
Flats, rocks and pilings	16
Rocks, pilings and dredging	10
Flats, pilings and dredging	3
Flats, rocks and dredging	9
Flats and under or on rocks	26
Wharfs and rocks	8
Flats and rocks	11
Dredging and rocks	5
Flats and pilings	2
Pilings and dredging	2
Flats and dredging	9
Flats, under rocks and dredging	4
On or under rocks	10
Pilings	7
Dredging	13
Only under rocks	1
Flats	41

The analysis shows that at the close of the 1920 season 54 species had been recorded from all four types of habitats studied while only 16 species were limited to and found in all of the three habitats excluding dredging. The flats have the greatest number of peculiar species, with 41, and but few forms are limited to any one of the other habitats.

The same material analyzed in another fashion is shown in Table 1. Here the attempt is to show the total numbers of animals found in each of the four major divisions of the littoral zones of the region in comparison with each of the other divisions.

TABLE I.

	Wharfs.	Flats.	Rocks.		Dredging.
Wharfs	110 100%	83 74%	80 73%	76 69%	(26 only in Harbor)
Flats	83 44%	187 100%	80[1] 43%	87 46%	(28 only in Harbor)
Rocks	80 72%	80 72%	111 100%	81 73%	(22 only in Harbor)
Dredging	76 64%	87 73%	81 69%	119 100%	(32 only in Harbor)

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[1] 30 more dug occasionally among or under rocks.

Again the analysis follows the records through the season of 1920. The comparison is based on total distribution records as was the last. That is, for the purposes of this table the finding of a given animal once in a given type of habitat is as significant as though it were abundant there. There were 242 species in the catalogue when this study was made.

It is immediately apparent that this type of analysis serves only to call attention to the larger number of animals taken from the flats and beyond indicating the higher specificity of the flats, shows no evidence of relationships that may exist between different habitats. The species taken from any given type of habitat are found to be approximately equally distributed in the other habitats of the region. Such experience has led casual observers to conclude that relationships between different animal associations can not be analyzed.

In order to make such an analysis, the records were studied from another angle. Species approximately equally distributed through the different associations and those reported for one season only were eliminated. Then the remaining species were listed according to the habitat in which they are most abundant. When a species was found equally abundant in two habitats it was listed from both. The association in which the animal was next most abundant was also estimated. Unfortunately these records are based on "experience" cards that seldom give specific figures and on the number of collecting teams that have reported the species from the different associations, as in the data given in the check list, rather than on strictly quantitative grounds and while substantially correct, they lack the finality that statistical treatment would give.

This type of analysis gives a real basis for comparisons of the relationship between the different associations. It is of interest that the relationships shown by Table II. are practically the same as given by an analysis of the entire catalog in 1917. In other words, the early collecting gave the typical forms characteristic of the environment while much of the later work has yielded in addition to these characteristic species, a number of accidental or incidental records.

TABLE II.

RELATION OF DIFFERENT HABITATS BASED ON DISTRIBUTION OF CHARACTERISTIC ANIMALS.

Name of Association.	No. Species.	Peculiar Species.		Species Found Next Most Abundant in: Pilings.		Rocks.		Flats.		Dredging.		Under or Among Rocks.	
		No.	%.	No.	%.	No.	%.	No.	%.	No.	%.	No.	%.
Wharf pilings......	32	2	6	—	—	24.5[1]	77	2.5	8	2.0	6	0	0
Rocks and rockweed	32	3	9	14.5	45	—	—	13	41	1.5	5	0	0
Dredging.........	20	3	15	3	15	9	45	3	15	—	—	0	0
Flats.............	40	22	25	2.5	3	15	17	—	—	17.5	20	30	33

The association of the wharf pilings is found to be closely related with that of the rocks; 77 per cent. of the animals common in the former are next most abundant in the latter. This fits with one's general impression that the two sets of animals are much the same but with the emphasis placed on different species so far as numbers are concerned.

The animals common on the rocks are next most abundant on the pilings but almost as many are nearly as abundant on the flats. The latter is to be expected from the fact that the rocks extend up from the flats, often forming a belt only a few feet wide in the intertidal portion of the flats, and that single rocks frequently occur surrounded by typical mud or sand flats, and from the further consideration that the eel grass offers almost as good a place of attachment for many animals as do the rocks.

The animals taken commonly in dredging are more closely related to the rock association than to the others. This is because our dredging operations have been done on clean hard bottom where the water conditions are similar to those found among rocks. Dredging in mud as in Buzzards Bay would give different results and transition bottom associations are indicated from the dredging work in Great Harbor.

Again the marked independence of the flats as a special habitat is shown by the larger number of animals frequently taken there in some abunbance and by the larger number of peculiar species. They are not closely related to other habitats if one excepts the conditions under the rocks which scarcely form a different association. Its main difference is that burrowing and exposure are more limited than on the open flats. The animals common on the flats are particularly absent from wharf pilings showing, as would be expected, that these habitats have little in common.

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[1] Where animals are approximately equally distributed between two different associations they are summarized by fractional representation.

If quantitative data were available, the distinctions here found would doubtless stand forth more plainly. The fact that a beginning can be made in indicating relationships by the methods used when no relationships appeared from an analysis of bare check lists emphasizes the need of quantitative studies in animal ecology.

This need was first recognized by Forbes (1907) who devised a mathematical formula for determining the existence of an association. In 1911, as the result of studying seasonal succession in ponds, I concluded that qualitative work gives insufficient basis for exact conclusions. Shelford in 1915 repeats the formula of Forbes, and Michael (16, 21) has done more than anyone else in America in showing the fundamental need of quantitative investigations in ecology and in developing formulæ to enable one to study associations on a quantitative basis.

The work here presented is of course only quasi-quantitative in character but the greater clearness obtained indicates that much of the muddle of animal ecology may be cleared by the further development and the application of quantitative methods in field researches. The problem of the ecologist studying littoral distribution is not so hard as that of the plankton student where as Michael says (1921): "Granting the equivalent of the oak tree or pine tree association, the marine ecologist finds difficulty not only in describing it but even in finding it. Since he cannot directly witness such an association, he is compelled to rely on indirect evidence furnished by tow-net or similar apparatus. In other words his only recourse is to measured magnitudes and application of mathematical logic thereto." For the exact determination of such relations the methods here used are almost as gross as are the ordinary qualitative observations in trying to solve the relationships existing between littoral associations.

It is true that analysis of the results of preliminary collecting showed the same relations as the quasi-quantitative analysis of more complete records in the Woods Hole region. Unfortunately one cannot be sure that the animals found in such preliminary work are really the typical animals since they may obviously contain many incidental forms. In other words in a random sample one is more apt to collect animals typical of the habitat than incidental forms but he can never be sure of this without further work.

V. The Effect of Continued Collecting on Distribution Records.

In 1917 when the collecting records were first studied seriously there were 181 species in the catalog. In 1920 when a similar study was made, the catalog listed 242 species. In the interim the Sound Gutters, Lackey's Bay and Great Harbor had been added to the localities visited.

In 1917, 11 species were recorded only from the wharf pilings. The later lists show 7 species so limited but this includes only one (*Tetrastemma*) of the previous list. In 1917 two species were recorded only from rocks or rockweeds, while on the later list there were 10 such species including only *Clava leptostyla* from the preceding list. In the first comparison there were 10 animals recorded only from dredging; in the later one, 13, which includes four of those on the preceding list: *Arca ponderosa*, *Strongylocentrotus droebachiensis*, *Heterocrypta granulata*, and *Amaroecium stellatum*.

On the 1917 list, 71 species were recorded from the flats only. After four more years' work this had shrunk to 41 providing animals found in the sand under and among rocks are excluded. Of these only 17 appear on both lists. They are: *Edwardsia elegans*, *Eloactis producta*, *Bdelloura candida*, *Syncoelidium pellucidium*, *Ophioderma brevispina*, *Chaetopterus pergamentaceous*, *Platynereis megalops*, *Scoloplos acutus*, *S. robustus*, *Spio* (*setosa?*), *Callianassa stimpsoni*, *Mysis stenolepis*, *Squilla empusa*, *Melampus lineatus*, *Clidiophora trilineata*, *Pecten irradians*, and *Tellina tenera*.

On the earlier lists, fifteen species were recorded from some place in each of the four main types of habitats: wharf pilings, rocks, flats, and dredging. In 1920 this list had increased to 54. These results mean, as has already been suggested, that as collecting has proceeded, animals have been picked up in habitats in which they are not abundant. The scarcity of many of these is shown by the number of single specimen records on the lists. There is little doubt but that if the present type of collecting were continued long enough, there would finally be stray records of many of the animals found in the region from each type of habitat. Even dredging, which we have usually carried on in deep water in Vineyard Sound, yields a different type of animals and becomes more closely related to other habitats as a result of dredging records taken in Great Harbor. In some one or more of their dredging operations, the *Biological Survey* found most of the animals we have taken from inshore digging. This result might be expected from the fact that some of their dredgings are recorded from less than 10 feet of water. If made at high tide, these would be almost as close inshore as our deepest collecting on wading and digging expeditions when we often collect out to four feet of water at low spring tides.

In other words, in such a small region as we are now considering, provided with strong tidal currents which aid in distribution, the animals tend to become widely distributed and occasional specimens will be found that can tolerate for a time conditions that are essentially unfavorable. Under these conditions the mere record of the presence of a species in a given habitat means very little unless there is due consideration of its abundance and duration in that locality. One is thus driven again to the conclusion of the last section, that quantitative work is necessary before final judgment can be passed in the matter of the constitution of animal associations.

We have found no evidence that the long continued collecting over the same grounds by the Invertebrate Class, nor the commercial collecting of the Supply Department of the M. B. L. has affected the number of animals present within the past nine years sufficiently for the effect to be noticeable by the collecting methods we have used. With growing experience in collecting each year we have broken previous records with monotonous regularity, for numbers of species from most of the localities we visit. This could not have continued so long had the animals been becoming less abundant.

The number of animals present in a given locality must depend more on the availability of suitable breeding places and abundance of food than upon such disturbing influences as summer collecting, particularly when the collecting does not reach all the breeding habitats of a region and there is adequate means of distributing young stages. This conclusion is emphasized by the rapid recovery in numbers of *Arbacia* after their almost complete disappearance following the winter of 1917–18 (Allee, '19) and in the face of their destruction by the thousands in the research work carried on in the Woods Hole laboratories.

VI. Summary.

1. Analysis of distribution records in the four major types of habitats of the Woods Hole littoral, viz., wharf pilings, rocks and rockweeds, flats, and the sea bottom in deeper water show that mere records of species present in the different habitats fail to indicate any relationship between the different types of associations.

2. By eliminating species known to be approximately equally distributed throughout and records for one year, only, and classifying the remaining species in terms of places where they are most abundant and next most abundant one finds:

(*a*) The association of the wharf pilings is closely related to that of the rocks.

(*b*) Species taken in dredging on clean hard bottom are found in next abundance on the rocks.

(*c*) The associations of the flats are highly independent of the others in the region but continue in the mud and sand under and around rocks.

(*d*) That some degree of quantitative work is necessary in order to determine the relationships of animal associations.

3. Preliminary collecting in a region tends to give the obvious forms and gives similar results in analysis to the type of quasi-qualitative work described in this report.

4. The number of animals present in the Woods Hole region has not been noticeably affected by the intensive collecting carried on there during the nine years covered by these studies.

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FOOD AND PARTHENOGENETIC REPRODUCTION AS RELATED TO THE CONSTITUTIONAL VIGOR OF *HYDATINA SENTA*.[1]

W. V. LAMBERT, W. S. RICE, AND H. C. A. WALKER.

There has been a general belief for many years that cross-fertilization is beneficial to a race. Darwin studied the effects of inbreeding and crossbreeding in many plants and came to the general conclusion that while inbreeding has a deleterious effect, crossbreeding has a beneficial effect upon the races involved.

In experiments with infusoria Calkins has found that if conjugation is prevented the race gradually weakened and finally died. He observed, however, that by the artificial stimuli of various chemicals in the culture water he was able to prolong the life of one race of *Paramecium caudautam* to the 742d generation.

Investigations in regard to the effects of parthenogenetic reproduction have been carried on by Shull and Whitney with the rotifer, *Hydatina senta*. Both of these investigators have found that continued parthenogenesis in pedigree races of rotifers has resulted in a gradual weakening and loss of vigor, and in some experiments death. In Whitney's experiments one race of rotifers died out from general exhaustion in the 384th generation and another in the 546th generation. A third race, discontinued in the 443d parthenogenetic generation had also shown a marked decrease in constitutional vigor.

Investigations of other workers, however, do not seem to confirm these general results. Woodruff, in his observations on the *Paramecium aurelia*, found that a race of these animals kept in cultures made from natural pond waters did not undergo marked depression or physiological changes. This race at the end of eight years, after having passed through 5250 generations without conjugation, was in a normal condition. Another race, however, subjected to the relatively constant hay infusion cultures generally employed, died after passing through several hundred generations.

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[1] Studies from the Zoölogical Laboratory, The University of Nebraska, No. 134.

King, also, in experiments with albino rats has found that continued inbreeding of the rat, when subjected to a varied and well balanced diet, does not weaken the race. On the contrary, the strains were larger, more fertile, and lived longer than many strains of stock rats in which no inbreeding was allowed. Castle, also, found after inbreeding the fly *Drosophila* (brother and sister matings) for fifty-nine generations, that no apparent decrease in the size and vigor, as compared to those with which he started, was noticeable. Noyes has recently finished the 250th parthenogenetic generation of the rotifer *Proales decipiens* and has detected no noticeable decline in vigor. Moreover, males of this species have never been seen. East and Jones are inclined to believe that if proper changes in the diet had been made in the experiments of Whitney and Shull upon the rotifer *Hydatina senta* that the normal vigor of the races would have been maintained.

PROBLEM.

The following experiments with the rotifer, *Hydatina senta*, were begun in order to determine if possible whether the gradual loss of vigor is due to the parthenogenetic reproduction or to a too restricted diet. The various races used in these experiments were kept under identical conditions as to temperature, light and culture water. Race *A* was fed a colorless *Polytoma* diet, whereas race *B* was fed a diet consisting of the *Polytoma* plus several kinds of green protozoa, predominately *Chlamydomonas*. The plan has been to keep the races in a parallel series and to study the effect of the two diets upon the two races. A third race (*C*) which has been obtained from wild rotifer cultures in the natural environment has been introduced into the series in order that the rate of reproduction of the rotifers under restricted and controlled environmental conditions might be compared with the rate of reproduction of those under the uncontrolled conditions of nature.

Acknowledgment is due Professor David D. Whitney for his suggestions and supervision of the work.

METHODS.

Some dried fertilized eggs of the rotifer, *Hydatina senta*, were collected at Lincoln, Nebraska, on September 17, 1920. One of these eggs hatched on September 29, and on October 1 two parthenogenetic sisters were isolated. One became the mother of what has been called race *A* and the other became the mother of what has been called race *B*. The two sister parthenogenetic races *A* and *B* were kept in syracuse watch glasses. Usually once in forty-eight hours five daughter-females of each race were isolated, each daughter-female being placed in a separate watch glass. They produced the young females of the succeeding generation. Race *A* was fed on a pure culture of the flagellate, *Polytoma*. This food was grown in horse manure solutions, which was prepared as follows: eight hundred cubic centimeters of fresh horse manure and one thousand cubic centimeters of tap water steam sterilized for one hour. This was cooled and diluted in the proportion of one part horse manure solution to two parts of boiled rain water. *Polytoma* were then put into it in an open pan and placed at about 18° C. The *Polytoma* grew very quickly and in 24–48 hours immense numbers of them were produced. This culture, ranging from 24–96 hours old, was decanted and replenished daily—one part culture medium to two parts boiled rain water being added. From the surface of this culture the *Polytoma* were taken with a sterilized pipette. This food was centrifuged so as to make it concentrated enough so that one drop was sufficient for each watch glass containing a female rotifer and a small amount of filtered rain water.

Race *B* was fed both from the pure culture of *Polytoma* and from two or three cultures of green flagellates, *Chlamydomonas*, *Chloroganium euchlorum*, and *Euglena*.

As some of the green food grew in salty cultures it was always washed with rain water before being placed in the watch glasses as food for the rotifers.

During the whole period of fifteen months the two races have been conducted in parallel generations. The external factors and environment have been as near alike as it has been possible to make them. The individuals of each generation were isolated at the same time, were put into the same kind of watch glasses and with approximately the same amount of filtered tap water, the only difference being in the food. The watch glasses were stacked side by side. During the day the room was lighted by the north light.

The method used for deciding whether the races had maintained their original vitality or whether they had decreased in their constitutional vigor has been the rate of parthenogenetic reproduction. In order to determine the comparative vigor of the races *A* and *B*, their rates of parthenogenetic reproduction were obtained by counting the young produced by a mother during a certain period of time for several successive generations at intervals of about thirty days.

The first comparison was made by counting the young daughter-females of both the races *A* and *B* from generations 22–29. The second comparison was made by counting the young in the same manner as above from the generations 43–47. The third was made by counting the young from generations 61–67. No green food was given to race *B* during the generations when these counts were made so that conditions would be identical at the time the rate of parthenogenetic reproduction was being determined.

During the above named generations fifteen daughter-females of approximately the same size of each race were isolated. This was done in order to give a greater number from which to get the average of young females produced, thereby decreasing the chance of error.

When the third and also the last comparisons were made fifteen females from various wild races of *Hydatina senta* were isolated and placed in separate watch glasses. These wild females have been called race *C*. This race was fed only pure culture of *Polytoma*. It was kept for only a few generations, being conducted under identical conditions and parallel to generations 65–67 and 169–175 of races *A* and *B*. This introduction was made in order to make a comparison of the rate of parthenogenetic reproduction between races *A* and *B* and various wild races taken from their normal environment.

RESULTS.

The first series of observations on the rates of parthenogenetic reproduction in these two races was started at the beginning of the twenty-second generation. The results at this time as can be seen in Table I., are extremely variable. Only five daughter-

TABLE I.

No. of Partheno-genetic Generation.	Date of Isolation.	No. of Young Females Isolated and Reproducing.	Period of Growth and Reproduction.	Race *A*. Av. No. of Daughters Produced.	Race *B*. Av. No. of Daughters Produced.
22........	11/16/20	5	48 hrs.	9.22	10.25
23........	11/18/20	5	48	7.20	5
24........	11/20/20	5	48	4	1
25........	11/22/20	5	48	7.8	1.4
26........	11/24/20	5	48	1.5	1.5
27........	11/26/20	5	72	4.2	4.5
28........	11/29/20	5	72	13.8	15.66
29........	12/2 /20	5	48	7	7.2
Average ..		5	54 +	6.84	5.81

females were isolated every other day for making the counts on the two races. The average number of young daughter-females produced in each forty-eight hour period at this time was extremely variable. The mothers of the *A* race during this period produced an average of 6.84 daughter-females while those of the *B* race produced an average of only 5.81.

The two parthenogenetic races were then carried on parallel with each other until the beginning of the forty-third generation at which time another series of observations was started on the number of young daughter-females each mother would produce in a given length of time, approximately 48 hours. As new generations were isolated great care was taken to pick uniform-sized daughters. One investigator did all of the isolating during this series of counts in order to insure the greatest uniformity. Considerable difficulty was experienced during this series of observations because of the large per cent. of male-producing females. These had to be discarded for only the mothers producing daughter-females were considered in calculating the averages.

The *A* race during this second series of observations produced an average of 7.58 daughter-females while the *B* race produced 8.88. Considerable variation occurred throughout the different generations, but the variations were less in this instance than they were in the preceding series of observations. A complete record of the results of this observation can be seen in Table II.

The two races were then carried on for fourteen generations before another series of observations was begun. At the begin-

TABLE II.

No. of Partheno-genetic Generation.	Date of Isolation.	No. of Young Females Isolated and Reproducing.		Period of Growth and Reproduction.	Race *A*. Av. No. of Daughters Produced.	Race *B*. Av. No. of Daughters Produced.
		A.	*B*.			
43..........	1/ 6/21	5	5	48 hrs.	7.4	10
44..........	1/ 8/21	10	11	48	6.6	3.8
45..........	1/11/21	14	13	48	6	10.8
45..........	1/11/21	5	5	48	6.5	6.9
46..........	1/13/21	8	11	48	12	15.2
47..........	1/15/21	15	14	52	7	6.6
Average.....				48.7	7.58	8.88

ning of the 61st generation fifteen individuals from each race were isolated as before and placed in separate watch glasses,—one person doing all the isolating as in the preceding series. No male-producing females appeared in this series of observations and the number of young produced in a given length of time was more nearly uniform throughout than was the case in the two preceding observations. The average number of young produced by the *A* race in the last three generations of this series of counts was 2.66 in an average of 46.3 hours. In the *B* race for the three generations an average of 3.68 daughter-females were produced in the same period of time.

TABLE III.

No. of Partheno-genetic Generation.	Date of Isolation.	No. of Young Females Isolated and Reproducing.			Period of Growth and Reproduction.	Av. No. of Daughter-Females Produced.		
		A.	*B*.	*C*.		Race *A*.	Race *B*.	Race *C*.
61.......	2/13/21	15	13		48 hrs.	4.84	5.84	
62.......	2/15/21	15	15		41	4.64	5.44	
63.......	2/17/21	15	15		47	2.90	3.37	
64.......	2/19/21	15	15		48	4.25	4.37	
65.......	2/21/21	13	15	15	44	1.15	2	2.33
66.......	2/23/21	15	14	15	48	3.56	4.21	4
67.......	2/25/21	5	14	14	48	3.80	5.21	4.25
Average..					46.3	2.66	3.68	3.53

At the beginning of the sixty-fifth generation, counts on the wild race of rotifers, race *C*, were started. They were reared under identical conditions with the *A* and *B* races and the counts were carried over a period of three generations. The average number of young produced by these mothers in 46.3 hours was 3.53. A complete record of the results of this observation can be seen in Table III.

The two parthenogenetic races were then carried on parallel with one another until the beginning of the 79th generation at which time a fourth series of observations was started.

During this fourth series of observations the *A* race produced an average of 7.46 daughter-females while the *B* race produced 8.29. Very little variation occurred throughout the different generations during this series of observations. A complete record of the results of this observation is given in Table IV.

TABLE IV.

No. of Parthenogenetic Generation.	Date of Isolation.	No. of Young Females Isolated and Reproducing.		Period of Growth and Reproduction.	Av. No. of Daughter-Females Produced.	
		A.	*B.*		Race *A.*	Race *B.*
79..........	3/22/21	15	15	48 hrs.	10.06	11.46
80..........	3/24/21	15	15	48	7.06	7.53
81..........	3/26/21	13	14	54	3.92	4.28
82..........	3/28/21	15	15	46	6.53	7.26
83..........	3/30/21	15	15	48	9.73	10.93
Average.....				48.8	7.46	8.29

The two races were then carried on for thirteen generations before another series of observations was made. The average number of hours between counts in this series of observations was 65.33 hours, the increase in length of time being due to the slowness of reproduction which was probably caused by the low temperature of the laboratory. The average number of young daughter-females produced during this series by race *A* was 3.32, while the number by race *B* was 5.15. A complete record of the results of this series is given in Table V.

The two races were again carried on parallel with one another until the beginning of the 106th generation. At this time they showed a very great degree of uniformity between the different

TABLE V.

No. of Parthenogenetic Generation.	Date of Isolation.	No. of Young Females Isolated and Reproducing.		Period of Growth and Reproduction.	Av. No. of Daughter-Females Produced.	
		A.	*B.*		Race *A.*	Race *B.*
96..........	4/28/21	14	15	77 hrs.	4.13	6.53
97..........	5/ 1/21	13	15	70	2.23	4.33
98..........	5/ 4/21	15	15	50	3.60	4.60
Average.....				65.33	3.32	5.15

generations, but there was a slight decrease in the difference between the number of young daughter-females produced by the two races. The *A* race during this series of observations produced an average of 9.19 daughter-females while the *B* race produced 10.06. A complete record of the results of this observation is given in Table VI.

TABLE VI.

No. of Parthenogenetic Generation.	Date of Isolation.	No. of Young Females Isolated and Reproducing.		Period of Growth and Reproduction.	Av. No. of Daughter-Females Produced.	
		A.	*B.*		Race *A.*	Race *B.*
106.........	5/23/21	14	13	48 hrs.	9.28	10.07
107.........	5/25/21	14	14	48	8.14	10.64
108.........	5/27/21	15	15	48	6.60	7.20
109.........	5/29/21	15	15	48	11.46	11.46
110.........	5/31/21	13	11	50	10.46	10.91
Average.....				48.4	9.19	10.06

The parthenogenetic reproduction of the parallel races was continued over the summer of 1921 without any further observations. The usual care was employed in their feeding. About the middle of August a wild race, *C*, was introduced. The seventh series of observations was begun with the 169th generation of the *A* and *B* races and the 10th of the wild race *C*. Considerable variation was found in the number of young produced by successive generations of each race. The difference, however, in the number of young of the *B* race as compared to the numbers of young of *A* and *C* was marked. The average period of growth and reproduction was 63.6 hours, during which period the average number of young of the *A* race was 6.86; of the *B*, 9.35; and of the *C*, 8.66. The complete tabulation of results may be found in Table VII.

TABLE VII.

No. of Parthenogenetic Generation.	Date of Isolation.	Number of Young Females Isolated and Reproducing.	Period of Growth and Reproduction.	Average No. of Daughter-females.		
				A.	*B.*	*C.*
A *B* } 169 *C*—10	10/ 5/21	10	72 hours	9.4	14.1	14.9
A *B* } 170 *C*—11	10/ 8/21	10	66 hours	9.8	10.1	10.33
A *B* } 171 *C*—12	10/11/21	10	56 hours	6.5	9.4	7.9
A *B* } 174 *C*—15	10/22/21	10	54 hours	1	2.75	1.7
A *B* } 175 *C*—16	10/25/21	10	70 hours	7.6	10.4	8.5
Averages		10	63.6 hours	6.86	9.35	8.66

The three races were then carried on side by side. Since the 145th generation the green food given Race *B* has been made up for the most part of *Euglena*, in place of the *Chlamydomonas* heretofore used. About the same time as the change in the green food the centrifuging of the *Polytoma* culture was discontinued and more drops of the unconcentrated food given instead. After the seventh series of observations, the investigators began to encounter difficulty with the supply of *Polytoma*. However, all three races of rotifers continued to reproduce parthenogenetically, although with slightly less than usual vigor in Race *A*. Nevertheless, notwithstanding all care possible in feeding, isolation and temperature the *A* race, fed only *Polytoma*, died out with the 198th generation, probably due to the faulty

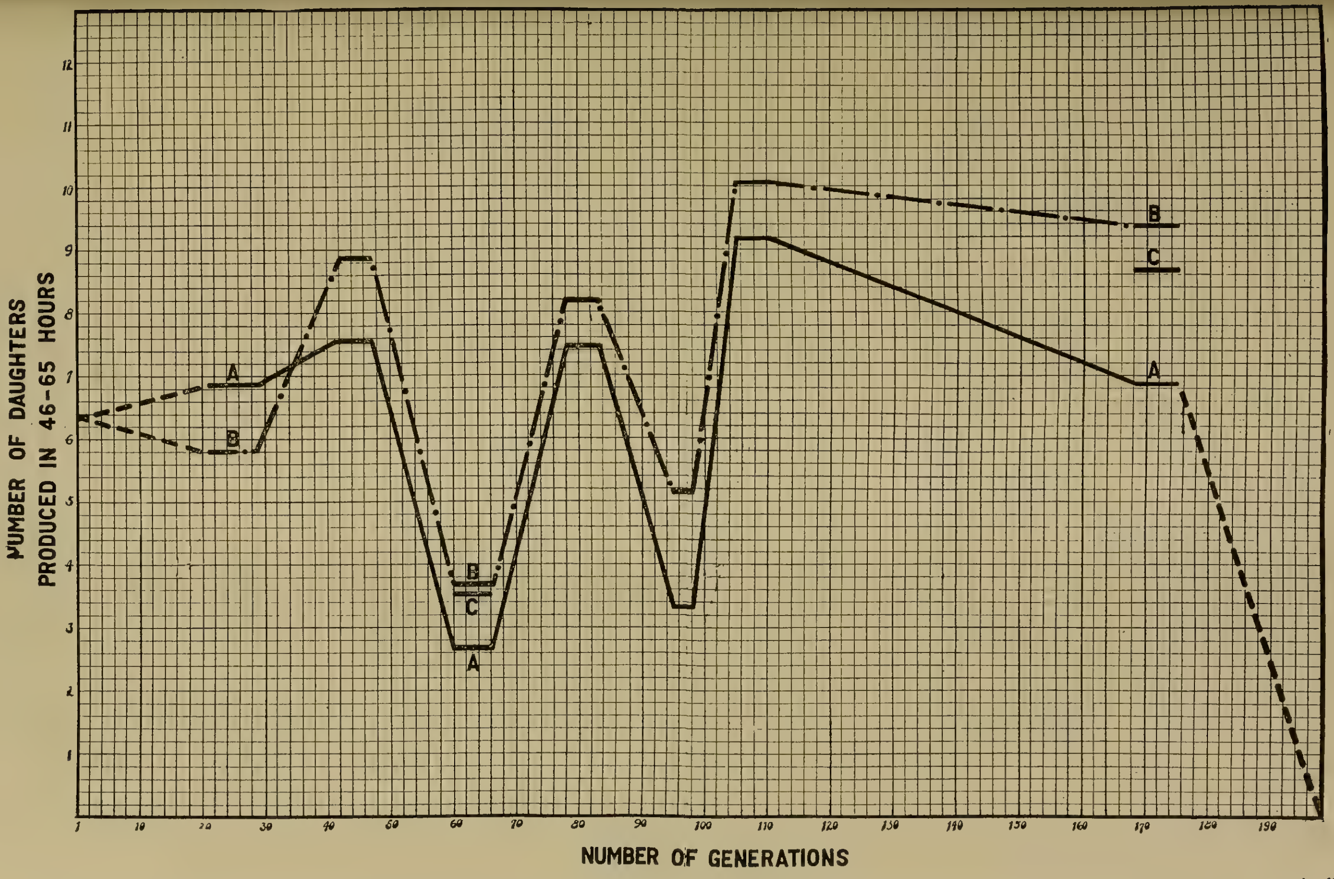

FIG. 1. Graphic representation of the results obtained from parallel races fed upon different diets and reproducing parthenogenetically. The unbroken line traces the history of Race *A*; the broken, of Race *B*; the other, of the wild races *C*, introduced at intervals for comparison. The lines from generation 1 to 22 and 176 to 198 are hypothetical, since no tests were made during these periods. The rate of reproduction of Race *A*, which was kept on the pure *Polytoma* diet, gradually declined, whereas Race *B*, kept on a mixed diet, showed no decline in the rate of reproduction.

Polytoma culture. Race *B* continued to the 209th generation and *C* to the 43rd, without apparent loss of vigor, when the experiment was discontinued. The entire history of the two races, *A* and *B*, may be seen in Fig. 1. These two races started from two sisters and their rate of reproduction was determined at seven different periods ranging over about 13 months. For some unknown reason Race B showed a lower rate of reproduction at the first count, but at the second count the rate had risen above that of Race A, and furthermore, in all the subsequent counts, covering about a year, it remained above the rate of Race A. Toward the end the two rates began to diverge considerably due to the gradual weakening of Race A while Race B maintained its former rate of reproduction.

CONCLUSIONS.

From the fact that the *A* race died out before the 200th generation it is obvious that some unexpected influence must have entered, most probably poor food, since other cultures of *Hydatina senta* have reproduced four and five hundred generations parthenogenetically. Therefore, our results can hardly be called conclusive, but the opinion seems to be justified that the constitutional vigor of the *A* race, fed upon an exclusive colorless diet, was noticeably weakened, while that of the *B* race, fed upon a diet of *Polytoma* plus a green food containing carbohydrates, was sustained and even raised above that of the wild races. If this is true, as it certainly seems to be, it would appear that the decline in vigor and eventual death in the races observed by Shull and Whitney was due to an unbalanced diet, rather than to the parthenogenetic method of reproduction, as they concluded.

Zoölogical Laboratory,
University of Nebraska,
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CONTENTS

ALLEE, W. C. *Studies in Marine Ecology.* III, *Some Physical Factors Related to the Distribution of Littoral Invertebrates.* 205

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BIOLOGICAL BULLETIN

STUDIES IN MARINE ECOLOGY: III. SOME PHYSICAL FACTORS RELATED TO THE DISTRIBUTION OF LITTORAL INVERTEBRATES.

W. C. ALLEE,

Marine Biological Laboratory and the University of Chicago.

PAGE
I. Purpose and scope of the investigation 205
II. Methods 208
III. Series of associations 209
  1. Associations of rocky eroding shores 210
    a. The open water association 210
    b. The wharf piling association 211
    c. The exposed rock association 215
    d. The protected rock association 219
  2. Associations of depositing shores 225
    a. The open water association 225
    b. The sand bar association 225
    c. The muddy sand association 227
    d. The eel grass and muck associations 228
      (1) The *Cumingia* association 229
      (2) The *Scolopos acutus* association 229
    e. The marginal muck associations 232
    f. The intertidal associations 234
      (1) The *Mya* association 234
      (2) The *Melampus* association 236
IV. Summary of data 236
V. Discussion 242
VI. Bibliography 248

I. Purpose and Scope of the Investigation.[1]

The first two papers in the present series give the results of a general faunistic ecological survey of the Woods Hole littoral region which occupied the summers of 1913–1921 inclusive. The records given there show the distribution of the common littoral invertebrates and discuss the relationships of the different animal associations present on the basis of their animal components.

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[1] I am indebted to the Marine Biological Laboratory for facilities for carrying on this survey and particularly to Mr. G. M. Gray for arranging for my comings and goings. I am also indebted to Dr. P. H. Mitchell of the Bureau of Fisheries for the loan of certified hydrometers and to Supt. W. H. Thomas for access to manuscript notes of daily temperature and salinity determinations.

The present paper deals with the results of an intensive study of some of the localities considered in the preceding sections, which was carried on in August and early September of 1920, with some additional data from records taken the following summer. In addition to considering the direct effects of different types of bottom and shores, currents, tides and vegetation, this study is particularly concerned with the possible correlation of temperature, salinity, oxygen content and pH with the intertidal and upper adtidal animal associations of the region immediately around Woods Hole.

Reference to the map published in connection with Study I. of this series will give the general location of the region and the *Biological Survey*[1] (pp. 170–190) gives a good account of the general physiographie features. The more important of these for the purpose of our discussion are:

1. The whole region lies far back from the edge of the continental shelf. The 20 fathom line runs 10 miles off Martha's Vineyard while the 100 fathom line, marking the edge of the continental shelf, is 75 miles from Gayhead and about 90 miles from Woods Hole.

2. The unequal height of the tides in Buzzards Bay and Vineyard Sound combined with the time difference of tides in the two bodies cause strong currents to flow almost incessantly from one to the other. All the localities discussed in this paper are directly affected by these tidal currents except the wharf pilings at Vineyard Haven and the bay at Gansett.

3. There is a general westerly drift in the region so that the *Biological Survey* estimates that all the water in Vineyard Sound is renewed weekly and in Buzzards Bay in about double that time.

4. The temperature is influenced by the Gulf Stream although his is normally shut from shore by a wall of cold water. The water immediately around Woods Hole is warmer in summer

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[1] Sumner, Osburn and Cole: A Biological Survey of the Waters of Woods Hole and Vicinity. Bul. Bur. Fish., Vol. 31.

and probably cooler in winter than outlying waters.[1] The *Biological Survey* reports an annual temperature range of 25° C. at the Fish Commission pier where the water is much mixed by the current through the Hole. The range is greater in stagnant water such as that in Blind Gutter at Lackey's Bay where the annual range is over 33° C.[2]

5. The intertidal zone is comparatively narrow, being more extensive in Buzzards Bay than in Vineyard Sound.

6. The shores of the region are usually lined with rocks which extend well below the low tide level and give way there to sandy bottoms in the more exposed places. In sheltered regions mud of organic origin is deposited over the sand.

7. The *Biological Survey* found that the salinity of Buzzards Bay averaged 3.00 per cent.; that of Vineyard Sound, 3.075 per cent. The open Atlantic runs to 3.6 per cent. or more.[3]

During the investigation the open water was sampled in four inshore localities:[4] Off the Buzzards Bay entrance to N. W. Gutter (Map, No. 10), in the Hole at Woods Hole; in Vineyard Sound at the entrance to Lackey's Bay (Map, No. 6) and at the entrance to Gansett Bay (Map, No. 11).

The wharf pilings were studied at Crane's Wharf (Map, No. 5) near the steamboat wharf in Woods Hole and at the old steamboat wharf in Vineyard Haven (Map, No. 1). The rocks at Northwest Gutter near its Bay entrance (Map, No. 10), in Lackey's Bay at the entrance to Blind Gutter Flats (Map, No. 6) and at Gansett. The associations of the flats were studied at Northwest and Blind Gutters and at Gansett, together with some observations in the creek leading to Southwest Gutter. In all 122 sets of collections were made.

These localities were chosen for intensive study because they are typical of the region and because of their proximity to and ease of access from the laboratory.

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[1] Cf. discussion in Biological Survey, pp. 37 and 50.

[2] Water over the eel grass in Lackey's Bay had a temperature of 32 deg. C. on the afternoon of August 15, 1920. There is no reason to suppose that this temperature is unusual. In winter the temperature must frequently fall below the freezing point of sea water.

[3] See Murray and Hjort.

[4] For descriptions of the different habitats see Study I. of this series.

II. Methods.

The water collections for oxygen samples were usually made according to the method given in Standard Methods for Water Analysis.[1] Where this method was impracticable, as in surface collections, the sample was taken by means of a two-valve rubber bulb. In all cases more than four times the amount of water necessary to fill the sample bottle was run through it before the sample was taken.

The oxygen determinations were by the Winkler method. The chemicals were added and the titrations were made in the field.

Salinity was ascertained by the specific gravity method with standardized bulbs loaned by Dr. Mitchell of the U. S. F. C. The salinity is expressed in the tables as calculated from the specific gravity according to the data given by True (1915).

The pH was determined colorimetrically by comparison on the spot with standard colors in tubes specially prepared for salt water by Hynson Westcott and Co. Two indicators were used mainly: Thymolsulphonephthalein (thymol blue) with a color range from 8.0 to 9.6 and dibromthymolsulphonephthalein (bromthymol blue) ranging from 6.0 to 8.0. These sets of standard colors were made up in June before they were used in August.[2] Doubtful determinations in the 7.7–8.0 range were tried with both indicators and on occasion with phenolsulphonephthalein, range 6.6–8.6. This last set of standard colors were made up in distilled water. Such readings were corrected for sea water by subtracting 0.1 from the observed reading.[3] Comparative readings with this correction applied are given in Table I.

The water for pH determinations was collected in a new 40 c.c. injecting syringe with a tight-fitting piston, either directly from the surface or from the large container of the standard apparatus used in collecting oxygen samples. This water was also used in finding temperatures from some depth below the surface. All determinations were ordinarily made in duplicate and, oxygen excepted, frequently in triplicate.

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[1] Report of the Committee on standard methods of water analysis. Jour. Inf. Diseases, Suppl. No. 1, pp. 1–141.

[2] The dibromthymolsulphonephthalein set was loaned me by Dr. E. B. Powers in 1920 and by Miss Myra Sampson in 1921.

[3] Atkins ('22) reports the salt error to be almost double that found here.

TABLE I.

SHOWING COMPARATIVE RESULTS OF USING THREE INDICATORS IN DETERMINING THE pH OF SEA WATER.

Indicator.	I.	II.	III.	IV.	V.
Dibromthymolsulphonephthalein......	8.0 +	8.0 +	8.0 +	7.3	8.0 +
Thymolsulphonephthalein...........	8.0 +	8.0 +	8.2	8.0 −	8.2
Phenolsulphonephthalein...........	8.0 +	8.0 +	8.2	7.3	8.2

III. SERIES OF ASSOCIATIONS.

The animal associations of the Woods Hole littoral can be divided into two series either on the basis of the character of the bottom, or of physiographic action. On either basis the resulting series are the same, for the eroding shores of the region are all rocky and the depositing shores have a shifting bottom composed of mud or sand. Within these series the associations may readily be arranged in the order of their physiographic age.

The associations of the rocky eroding shores as studied are: (1) open water; (2) wharf pilings; (3) exposed rocks; (4) protected rocks. The associations of the depositing shores likewise begin with (1) the open water and typically continue as follows: (2) those of the sand bar; (3) with the deposition of mud this becomes the muddy sand association in which eel grass begins; (4) with further deposition of mud and as the eel grass grows longer one finds the *Cumingia* association and in deeper water where still more muck has been deposited, the *Scoloplos acutus* association. These make up the two main associations of the eel grass and muck, which extend to the margin of the eel grass in about a foot of water at low tide. Between this level and the shore at about low tide lies the marginal muck association (5) which gradually gives way to the intertidal associations, the lower of which may be called the *Mya* association and occupies the region from just below low tide to about the half tide mark. The upper association is marked by the abundance of the snail *Melampus* and may well be called by that name. In places the *Ucas* are more abundant, but such localities have not been studied in this report.

In order to understand this typical arrangement of associations in the depositing shore series it is necessary to know that in the flats of the region there is usually a sand bar formed at the outer edge of the flat which is frequently uncovered at low tide; farther back on the flats the water becomes slightly deeper in the region of mud and sand and then decidedly deeper until one reaches the *Scoloplos acutus* association of the eel grass and muck region. As one nears the shore the water becomes shallow again.

While the series as given is typical of the region there are several other possibilities which will be discussed in their proper places.

1. *The Rocky Eroding Shore Associations.*

a. The Open Water Association.—The results of analyses of water samples from four inshore stations are shown in Table II.

TABLE II.

SHOWING SALINITY, TEMPERATURE, OXYGEN CONTENT AND pH OF OPEN WATER FROM INSHORE STATIONS.

Date.	Time.	Depth Ft.	Tide.	Temp. ° C.	pH.	O. in c.c. per L.	Salinity %.	Light.	Coll. No.
					I.				
1920 8/16	10:50	14	High	21	8.0	—	—	Dull	11
	11:10	22	High	21	8.0	3.93	—	Dull	12
9/3	9:15	22	Do.	20	8.0 −	4.37	3.02 3.05	Bright	107[1]
					II.				
8/16	4:00	15	Sound to Bay	22	8.0 +	—	—	Dull	22
	4:10	10	Do.	22	8.0	—	—	Dull	23
1921 9/12	11:45	12	Do.	21	8.0	5.43	3.14	Dull	122
					8.0	5.38	3.14		
				21	8.0	5.38	3.14		
					8.0	5.38	3.14		
				21	8.0	5.35	3.14		
				21	8.0	5.38	3.14		
				21	8.0	5.35	3.13[2]		
					8.0	5.33	3.12[2]		
					8.0	5.38	3.12[2]		
					8.0	5.38	3.12[2]		
					III.				
1920 9/3	5:00	12	Bay to Sound	21	8.1	—	3.04	Bright	120
					IV.				
8/31	4:10	24	Low	22	8.2	6.14	—	Rainy	100
	4:10	24	Low	22	8.0	5.84	3.09	Rainy	100′

[1] Collection 7 feet down.

[2] Determination in laboratory.

[3] Collection made 12 feet down.

Part I. is from collections made off the Bay opening of Northwest Gutter about 75 yards directly west of the corner of the channel made by the shoulder of Uncatena Island. Part II. is from the passage in Woods Hole and the third part is from the opening of Lackey's Bay into Vineyard Sound. The latter is influenced by the current carrying water from the extensive submerged vegetation of this region. Part IV. is from the mid-mouth of Gansett Bay. The surface water here also shows the effect of proximity to vegetation. The depths are those given in the U. S. Coast and Geodetic Survey charts. The last two divisions are given for comparison only and will not be considered in discussing open water conditions since they are obviously atypical.

The invertebrate animals to be found near the surface in such locations are largely plankton. A typical collection taken with miller's bolting cloth net from a Bay tide in Woods Hole at 10:00 A.M., August 8, 1917, is shown in List 1. The collection was studied the afternoon after it was taken by the class in Invertebrate Zoölogy of the Marine Biological Laboratory with the assistance of seven instructors. The list is based on the findings of approximately sixty people in a two hours search.

LIST 1. SHOWING THE MORE CONSPICUOUS ANIMALS TAKEN IN ONE TOWING IN THE HOLE.

Animals.	Number Found.
PROTOZOA	
Ceratium	8
CŒLENTERATA	
Actinula of *Tubularia*	2
Hydromedusæ	12
NEMERTINI	
Pilidium of *Cerebratulus*	1
CHÆTOGNATHA	
Sagitta	2
ECHINODERMA	
Bipinnaria	12
Brachiolaria	5
Pluteus	2
ANNELIDA	
Dinophilus	5
Trochophores	2
BRYOZOA	
Cyphonautes	5
CRUSTACEA	
Nauplius	3
Zœa	5
Mysis	4
Megalops	5
Copepoda	∝
Cypris larvæ of *Balanus*	1
MOLLUSCA	
Lamellibranch veligers	4
Gasteropod veliger	∝
CHORDATA	
Appendicularia	14
Botryllus tadpoles	1

b. Wharf Piling Association.—The animals in this association, whether free living, attached, or living among attached forms, are more abundant at the outer end of the piers where the water is deeper and the wave action is less violent. The conditions in the water, so far as investigated, are given in Table III. It will be noted that the pH was constant in all locations; that the oxygen content was relatively constant for each location, with the exception of one collection taken at Crane's Wharf just after the passing of a Nantucket steamer which caused a decided increase. There is no evidence of vertical gradients among the factors analyzed to explain the vertical distribution of animals found on the wharf pilings; nor is there an indication of horizontal gradients along the wharf save in salinity.

Table III.

Showing Salinity, Temperature, Oxygen Content and pH of Water from Wharf Pilings Association.[1]

Date 1920.	Time.	Depth in Ft.		Temp.	pH.	O_2 in c.c. per L.	Salinity.	Collection Number.
		Water.	Collection.					
8/21	8:30	12 +	12	19	8.0	4.48	—	40
	8:45	12 +	1	19	8.0	—	—	40
	9:20	10	10	20	8.0	—	2.97	41
	9:40	1	1	20	8.0	—	—	42
	10:10	12 +	0.25	—	—	6.0	—	43
8/22	9:40	12 +	1	20	—	4.36	—	44
	9:40	12 +	1	20	—	4.26	—	45
	10:00	12 +	12	20	—	4.16	—	46
	10:00	12 +	12	20	—	4.26	—	47
	10:35	12 +	1	—	—	4.11	—	48
	10:35	12 +	12	—	—	4.06	—	49
8/28	3:30	1	1	21	8.0	5.25	3.01	85
	3:45	12 +	12	20	8.0	5.25 5.15	3.07	86
	4:00	12 +	1	21	8.0	5.25	3.13	87

All the animals found in collecting on Crane's Wharf pilings are given in List 2. The figures give an arbitrary means of showing relative abundance and are based on the number of collecting teams reporting each animal in one afternoon's work early in July, 1920. Absence of such figures shows that the animals were not found at that time.

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[1] Low tide and bright sunlight throughout.

LIST 2. SHOWING THE INVERTEBRATE ANIMALS TAKEN FROM CRANE'S WHARF PILES IN NINE YEARS' COLLECTING.

The figures give an indication of comparative abundance in 1920 and are based on the number of collecting teams reporting the several species. Absence of figure indicates that the animal was not taken during this trip.

	Sessile	Free
PORIFERA		
Chalina arbuscula	7	
Cliona celata(?)	7	
Grantia ciliata	7	
Leucosolenia botryoides	5	
Microcione prolifera	7	
CŒLENTERATA		
Campanularia sp.	1	
Clytia bicophora	7	
Eudendrium album		
Eudendrium ramosum		
Gemmaria gemmosa	1	
Hydractinia echinata		
Metridium dianthus	6	
Obelia commissuralis	4	
Obelia geniculata		
Pennaria tiarella	1	
Sagartia luciæ	1	
Sagartia leucolena	1	
Schizotricha tenella		
Sertularia pumila		
Tubularia crocea	7	
PLATYHELMINTHES		
Tetrastemma vermiculum	1	
Lineus bicolor		
NEMATHELMINTHES		
Pontonema marinum	5	
ECHINODERMA		
Arbacia punctulata		3
Asterias forbesi		5
Asterias vulgaris		1
Henricia sanguinolenta		
ANNELIDA		
Arabella opalina		1
Amphitrite attenuata		2
Autolytus cornutus		1
Harmothœ imbricata		5
Hydroides hexagonus	5	
Lepidonotus squamatus		7
Lepræa rubra		
Nereis pelagica		7
Nicolea simplex		2
Podarka obscura		2
Polycirrus eximeus		3
Polydora sp.		
Phyllodoce catenula		
Sabellaria vulgaris		
Spirorbis spirorbis	6	
Sthenelais leidyi		
Thelepus sp.		
BRYOZOA		
Ætea anguina	5	
Bugula turrita	7	
Bicellaria ciliata		
Crisia eburnea	7	
Lepralia pertusa		
Membranipora pilosa	6	
Flustrella hispida	2	
Schizoporella unicornis	6	
ARTHROPODA		
Amphithœ rubricata		2
Autonœ smithi		1
Anoplodactylus lentus		2
Balanus balanoides		7
Balanus eburneus		2
Caprella geometrica		7
Erichsonella filiformis		1
Idothea baltica		2
Libinia dubia		6
Libinia emarginata		6
Lygidia oceanica		2
Orchestia agilis		
Palæmonetes vulgaris		
Palene empusa		1
Pinnotheres maculata		2
Panopeus sayi		6
Talorchestia longicornis		
Tanystylum orbiculare		2
MOLLUSCA		
Acmea testudinalis		1
Anomia aculeata	1	
Anomia ephippium	4	
Arca pexata		
Bittium alternatum		4
Busycon carica		
Columbella avara		6
Columbella lunata		7
Crepidula convexa		
Crepidula fornicata		3
Crepidula plana		3
Coryphella gymnota		5
Lacuna vincta		4
Littorina litorea		7
Littorina palleata		1
Littorina rudis		1
Modiolus modiolus	1	
Mytilus edulis	6	
Teredo navalis		3
Odostomia sp.		
Urosalpinx cinereus		7
CHORDATA		
Amarœcium constellatum	6	
Botryllus schlosseri		
Didemnum lutarium	7	
Molgula arenaria	1	
Molgula manhattensis	4	
Molgula papillosa	1	
Perophora viridis	6	
Styela partita	7	

Table IV. shows data taken from the records from the U. S. Fish Commission from their collections off the Fish Commission Pier and is given for comparison with records from wharf pilings and also with those from open water.

Table IV.

Showing Temperature and Salinity from the Noon Readings of the U. S. Fish Commission at their Woods Hole Wharf for August, 1920.

The raw data in sp. gr. and Fahrenheit have been converted to percentage of salinity and the centigrade scale for purposes of comparison.

Day.	Salinity.	Temperature.	Day.	Salinity.	Temperature.
1	3.20	21.7	16	3.22	22.2
2	3.19	20.8	17	3.30	22.5
3	3.205	21.1	18	3.285	21.9
4	3.18	20.6	19	3.19	20.8
5	3.215	20.0	20	3.185	20.0
6	3.24	20.3	21	3.20	20.6
7	3.25	20.3	22	3.215	20.1
8	3.24	20.6	23	3.235	20.6
9	3.235	21.9	24	3.235	20.6
10	3.24	22.2	25	3.215	21.1
11	3.22	21.7	26	3.21	20.8
12	3.24	22.2	27	3.205	21.1
13	3.24	22.2	28	3.21	21.4
14	3.24	22.2	29	3.21	21.4
15	3.21	21.7	30	3.04	21.7
			31	3.065	21.7

The vertical distribution in the wharf piling association is marked. Near the high tide level may be found the *Littorinas*, *Urosalpinx*, *Balanus balanoides* and an occasional *Asterias forbesi*. Nearer the water at low tide are *Mytilus* clusters and *Modiolus modiolus* sheltering *Libinia dubia* and *Lepidonotus*. Below these, normally under water at all times, come *Amarœcium* and *Styela* and other pile dwelling chordates. Here are found the sponges, *Microcione*, *Grantia* and *Cliona* and the hydroids such as *Tubularia* and *Schizotricha*. With all these there is the attendant fauna of worms, *Lepidonotus*, *Harmothoe*, *Sthenelais*, *Nereis pelagica* and *limbata;* the sea slug *Coryphella* and the *Columbella* snails are also abundant. Here also are *Bugula* and the encrusting bryzoans, *Schizoporella*, *Lepralia*, *Membranipora* and the like. Below these are the sea anemones, *Metridium*.

The animals mentioned may be displaced somewhat from the order given but as one looks down the outer piles at Vineyard Haven four distinct bands can easily be recognized: The *Balanus* band well above the low water level; the *Mytilus* band near low water, the *Amarœcium-Tubularia* band which shows as a more brilliantly colored region and finally the dull brown of the *Metridium* band which extends well down toward the bottom of the pilings.

Apart from the obvious relations in the upper portion where independence of water is the controlling factor, the vertical distribution may be in part the effect of light differences particularly on the free swimming larvæ. Grave ('20) has found that *Amarœcium* tadpoles, for example, are first positive but soon become negative to light. In part the position may be a response to gravity for the same tadpoles are at first negative in their reaction to gravity and later become positive. When both light and gravity act as they would in nature, the tendency of the tadpoles to settle to the bottom of the experimental cylinder was most marked and occurred in 85 per cent. of the cases. The banding may also be controlled in part by reactions to pressure and to conditions in the water that could not be detected by the measurements made. On the whole Grave is inclined to attribute the distribution of *Amarœcium* colonies to the effect of gravity during the active, free-swimming life of the tadpoles.

c. Rock Associations.—There are two distinct types of animal associations to be found in the region dominated by rocks. First, there are the animals and plants attached to the rocks and rockweeds or living among those so attached and, second, there are the animals living in the substratum under and sometimes between the rocks. These last are closely similar to the animals of the mud or sand flats near which the rocks are located. No attempt has been made to get water samples exactly characteristic of the second community, since in this work the main attention was focused on the animals of the rocks.

Two animal associations of the rocks proper are easily recognized. On the exposed rocks, where there is little rockweed, the animals are mainly found under the edges of the rocks out of the direct sweep of the waves. In protected regions, the rocks are covered with the rockweeds, *Ascophyllum* and *Fucus*. Here many of the animals are to be found on the upper surface of the rocks under the protecting rockweed. This association may be designated as the protected rock association or more obviously as the rockweed association.

(1) *Exposed Rock Association.*—The association inhabiting the exposed rocks was much studied at the opening of Northwest Gutter into Buzzards Bay. The rocks are exposed to the full sweep of the waves across the Bay and support a sparse growth of *Ascophyllum* and *Fucus*.

Similar conditions are common along the coast. They were studied in three localities: (1) At the outer point of East Buck Island in Lackey's Bay; this differs only in that submerged vegetation lies closer to the rocks on the shore side and extends around the rocks on the Sound side. The waves from the Sound break freely over these rocks. (2) At Gansett on the point which separates Gansett Bay from Quissett Harbor. The whole bottom nearby is covered with eel-grass while in the immediate vicinity *Sargassum filipendula*, *Ascophyllum* and *Fucus* grow in abundance. These rocks are frequently reached by the direct waves from Buzzards Bay and are themselves practically free of rockweeds. (3) In the region of Southwest Gutter where the rocks are protected from the sweep of the waves but are washed by strong and almost constant tidal currents coming now from the Sound and now from the Bay. The direction of the current makes little difference in the character of the water since in either case it passes through tortuous, shallow passages supporting a large amount of submerged vegetation. While there are many plants nearby, these rocks do not support a large amount of plant life. Water analyses from this association are given in Table V.

The animals most common in the exposed rock association are the sponges, *Microcione* and *Cliona;* the actinians, *Sagartia leucolena* and *luciæ* and *Metridium;* the starfish, *Asterias;* the annelids, *Harmothoe*, *Hydroides*, *Lepræa* and *Lepidonotus;* the bryozoan *Bugula* and various incrusting forms; *Balanus balanoides* and *eburneus*, the barnacles; *Chætopleura*, the chiton; the bivalve molluscs, *Anomia* and *Mytilus;* the snails, *Acmea*, *Littorina litorea*, *Eupleura*, *Purpura* and *Urosalpinx;* and the chordates, *Amaræcium*, which is usually and characteristically

TABLE V.

SHOWING OXYGEN CONTENT, pH, TEMPERATURE AND SALINITY OF WATER FROM THE ANIMAL ASSOCIATION OF THE EXPOSED ROCKS.

Date 1920.	Depth Water.	Tide.	Temp.	pH.		O_2.		Time.	Light.	Salinity.	Coll. No.	Place.
				Bot.	Sur.	Bot.	Sur.					
8.14	2 ft.	Flowing	21	8.1	—	—	—	4:30	Dull	3.04	9	Gutter (N.W.)
8.18	1 ft.	Ebbing almost low	21	8.1	—	3.62		3:50	Dull	—	37	
8.23	1 ft.	Low	20	8.0	—	4.41	—	9:00	Dull	3.04	50	
8.24	3 ft.	Flowing almost low	20	8.0	—	4.95	—	11:10	Bright	2.94	65	
9.3	7 ft.	2/3 in	20	8.0	—	4.25	—	10:00	Bright	2.99	108	
8.25	2 ft.	Low to bay	21 on surface	—	8.2	—	5.45	11:35	Bright	—	79	Gutter (S.W.)
8.25	6 ft.	Low to bay	21 on surface	—	8.2	—	6.54	12:45	Bright	3.01	81	
9.3	6 ft.	Slack	21 S.	—	8.2	—	5.39	2:30	Bright	3.01	116	
8.25	1.5 ft.	Low	22 S.	8.2	—	7.03	—	1:30	Bright	3.04	82	Lackey's Bay
9.3	1 ft.	Ebbing	21	8.2	—	4.16	—	4:40	Bright	3.04	120	
8.30	2 ft.	Low	23	8.3	8.3	8.56	—	2:45	Bright	—	89	Gansett
8.30	2.5 ft.	Flowing	22	8.3	—	8.41	—	3:45	Bright	—	91	
8.30	3 ft.	Flowing	—	—	8.3	—	—	4:10	Bright	—	93	
9.1	6 ft.	Near high	22	8.0	8.0	5.15	—	11:30	Overcast	3.05	96	
9.1	2 ft.	Low	21	8.2	—	6.14	—	3:45	Overcast	3.02	104	

LIST 3. SHOWING ANIMALS COMMONLY FOUND IN THE EXPOSED ROCK ASSOCIATION.

Animals starred may be taken above low tide mark.

PORIFERA
- *Cliona celata*(?)
- *Grantia ciliata*
- *Leucosolena botryoides*
- *Microcione prolifera**

CŒLENTERATA
- *Astrangia danæ*
- *Clava leptostyla*
- *Hydractinia echinata**
- *Metridium dianthus*
- *Sargartia leucolena*
- “ *luciæ**
- “ *modesta*
- *Sertularia pumila*

PLATYHELMINTHES
- *Stylochus ellipticus*

ECHINODERMA
- *Arbacia punctulata*
- *Asterias forbesi**

ANNELIDA
- *Harmothoe imbricata**
- *Hydroides hexagonus**
- *Lepræa rubra*
- *Lepidonotus squamatus**
- *Nicolea simplex*
- *Polycirrus eximeus*
- *Spirorbis spirorbis**
- *Sabellaria vulgaris**

BRYOZOA
- *Alcyonidium* sp.
- *Bugula turrita**
- *Crisia eburnea**
- *Flustrella hispida*
- *Lepralia pertusa*
- *Membranipora pilosa**
- *Schizoporella unicornis**

CRUSTACEA
- *Amphithoe*
- *Balanus balanoides**
- *Balanus eburneus*
- *Cancer irroratus**
- *Idothea metallica*
- *Idothea baltica**
- *Pagurus longicarpus**
- *Panopeus sayi**
- *Sphæroma quadridentatum**
  (Under rocks)

MOLLUSCA
- *Chætopleura apiculata*
- *Anomia ephippium**
- *aculeata*
- *Modiolus modiolus**
- *Mytilus edulis**
- *Acmea testudinalis*
- *Bittium alternatum*
- *Cerithiopsis emersonii*
- *Columbella avara*
- *lunata**
- *Coryphella gymnota*
- *Crepidula fornicata*
- *convexa*
- *plana**

- *Eupleura caudata*

- *Lacuna vincta**
- *Littorina litorea**
- *palliata*
- *rudis**
- *Purpura lapillus**
- *Odostomia* sp.
- *Urosalpinx cinereus**

CHORDATA
- *Amarœcium constellatum**
- *Botryllus schlosseri**
- *Didemnum lutarium*
- *Molgula manhattensis*
- *Perophora viridis*
- *Styela partita*

present in small clusters, and *Styela*. A more complete list is given in List 3, which while not exhaustive, does represent the association in an adequate manner. The species starred were taken above low spring tide in collecting on the rocks at Kettle Cove, August 4, 1921.

The chance of differentiation in the vertical distribution is not so great here as on the wharf pilings and, in addition, the spaces between the rocks and under them hold moisture better than in the case of the pilings so that the break at the tide level is less marked. Nevertheless the animals to be found on the rocks vary greatly with the height above mean low tide. Differences found in animal life and in water analyses at different tide levels are given in List 4 and Table VI. The high tide levels were studied on the Northwest Gutter rocks. The tidepool and region below low tide are from data obtained at Gansett. There is no reason to suppose that high tide conditions are different in the two places.

The tidepool was well separated from the water of the Bay. It contained about 6–12 inches of water and was one foot wide by three feet long. Some rockweeds were present but not in the numbers common in a typical rockweed association.

It is possible that the greater acidity combined with the decreased oxygen content of the water at the margin at high tide might cause sensitive larvæ to turn back. However when the waves are running well they would have no power in the matter. It is also possible that the sensitive animals of the exposed rock association are kept out of the tide pools by the great range in acidity which was over twice that found on the nearby rocks below low tide and, as seen in List 4, the animals present are fewer in numbers and in species.

(2) *Protected Rocks or Rockweed Association.*—The best example of the rockweed association to be studied was found in the creek between Southwest Gutter and Hadley Harbor. In this place the rockweeds, *Ascophyllum* and *Fucus*, form a patch about two rods square which reaches half way across the creek. The water ranges from six inches to four feet in depth. Between the rocks on the bottom are small patches of gravelly sand mixed with humus. Large boulders, which extend well out of the water at

TABLE VI.

SHOWING OXYGEN CONTENT, pH, TEMPERATURE AND SALINITY OF WATER OVER DIFFERENT TIDAL LEVELS OF MORE EXPOSED ROCKS.

Date 1920.	Depth of Sample.	Tide.	Temp.	pH.		O_2. Bot.	Light.	Time.	Salinity.	Coll. No.	Place.
				Bottom.	Surface.						
8/16	2 ft.	High	21	7.8	—	3.56	Dull	9:10	—	10	Gutter (N.W.)
				7.9	—	3.72					
8/18	1 ft.	Half	22	7.9	—	3.18	Rainy	9:30	—	24	
8/30	.7–1 ft.	Low	24	8.4	—	8.32	Bright	3:00	—	90	Tide pool Gansett
8/30	1.25 ft.	Flowing	24	8.4	—	8.02	Bright	4:00	—	92	
8/30	1.5 ft.	Flowing	23	8.4	—	8.02	Sunny	4:20	—	94	
9/1	3 ft.	Ebbing	22	7.7	—	5.15	Overcast	11:40	3.05	97	
9/1	.7 ft.	Flowing	22	8.0	—	5.94	Sunny	4:00	3.06	105	
8/30	2 ft.	Low	23	8.3	8.3	8.56	Bright	2:45	—	89	Below low tide Gansett
8/30	2.5 ft.	Flowing	22	8.3	—	8.41	Bright	3:45	—	91	
8/30	3 ft.	Flowing	—	—	8.3	—	Bright	4:10		93	
9/1	6 ft.	Near high	22	8.0	8.0	5.15	Overcast	11:30	3.05	96	
9/1	2 ft.	Low	21	8.2	—	6.14	Overcast	3:45	3.02	104	

LIST 4. SHOWING ANIMALS FOUND AT DIFFERENT TIDE LEVELS ON MORE EXPOSED ROCKS.

A. At extreme high tide; NW. Gutter rocks.

Orchestia agilis.
Talorchestia longicornis.

B. At Half Tide; Same Place.

CŒLENTERATA:
Sertularia pumila, much.
CRUSTACEA
Balanus balanoides, very abundant.

MOLLUSCA:
Mytilus edulis, several, small.
Littorina litorea, very many.
rudis, several.
palliata, some on *Ascophyllum*.

C. Tide Pool among Rocks, Gansett.	*D. Below Low Tide, Gansett.*
PORIFERA	
Cliona celata. (?)	Ten times as much as in *C*.
Microcione prolifera, some.	Much, more than in *C*.
	Leucosolenia botryoides, sev. colonies.
	Grantia ciliata.
CŒLENTERATA:	
Sagartia leucolena, few	Several
	Astrangia danæ, sev. colonies
	Metridium dianthus, several.
	Sertularia pumila.
BRYOZOA:	
Bugula turrita	More here.
	Crissia eburnia, 1 colony.
ANNELIDA:	
Harmothoe imbricata, several.	Several, more than in *C*.
Hydroides hexagonus	Many more than in *C*.
Lepræa rubra, few.	Many.
Lepidonotus squamatus	About same as *C*.
Spirorbis spirorbis.	
CRUSTACEA:	
Balanus balanoides, very many	Some
	Gammarus sp. many.
MOLLUSCA:	
Anomia ephippium, several small.	Large ones here.
Acmea testudinalis	More than in *C*.
Cerithiopsis emersonii	None.
Littorina litorea, very abundant.	Fewer than in *C*.
Urosalpinx cinereus	Many more than in *C*.
	Chætopleura apiculata, few large.
	Anomia aculeata, several small.
	Modiolus modiolus, few.
	Crepidula plana, few.
	Littorina rudis, few.
CHORDATA:	
Stylela partita	Few in both.
	Amaræcium constellatum, much.

low tide, support masses of the rockweeds. The water here is constantly changed by the strong tidal current.

A similar situation, except for the tidal current, was examined at the inshore end of Gansett Bay. The region there is in the form of a triangle with the shore for the long base and with the apex made by a large boulder lying about two rods off shore at low tide.

A partial list of the animals to be found in this association, divided according to strata, is given in list 5. As might be expected, animals usually found only at or near low tide, occur here well above low tide in some abundance on account of the protection furnished and the water held by the rockweeds.

List 5. Showing Representative Animals of the Rockweed Association by Strata.

A. Animals on or Among the Rockweed when Left Exposed by the Tide.

Cœlenterata:
- *Clava leptostyla,*
- *Obelia geniculata,*
- *Sertularia pumila,*
- *Sagartia luciæ.*

Echinoderma:
- *Asterias forbesi.*

Bryozoa:
- *Alconidium* sp.?,
- *Bugula turrita,*
- *Flustrella hispida.*

Arthropoda:
- *Amphithoe rubricata,*
- *Caprella geometrica,*
- *Balanus balanoides,*
- *Anoplodactylus lentus,*
- *Limulus polyphemus.*

Mollusca:
- *Mytilus edulis,*
- *Littorina litorea,*
- *Littorina palliata,*
- *Columbella avara,*
- *Columbella lunata.*

B. Animals from the Rocks Below the Rockweed Stratum from First Six Rocks Examined.

Porifera:
- *Cliona celata,* (?)
- *Microcione prolifera,*

Cœlenterata:
- *Sagartia leucolena.*

Echinoderma:
- *Asterias forbesi.*

Bryozoa:
- *Lepralia pertusa,*
- *Membranipora pilosa,*
- *Schizoporella unicornis.*

Annelida:
- *Harmothoe imbricata,*
- *Lepidonotus squamatus,*
- *Hydroides hexagonus,*
- *Polycirrus eximeus.*

Arthropoda:
- *Cancer irroratus.*

Mollusca:
- *Mytilus edylis.*

Chordata:
- *Styela partita.*

C. Animals Found in Brief Examination on Surface of Substratum.

CRUSTACEA:
- *Cancer irroratus,*
- *Libinia dubia* carrying the sponge, *Cliona celata,* (?)
- *Pagurus longicarpus* carrying the hydroid, *Hydractinia echinata,*
- *Panopeus sayi.*

GASTEROPODA:
- *Littorina litorea,* very numerous.

D. Animals Found in Sampling the Substratum. (Three spadesful were dug.)

PLATYHELMINTHES:
- *Lineus ochræus.*

MOLLUSCA:
- *Cumingia tellinoides,*
- *Nassa trivitatta.*

ANNELIDA:
- *Clymenella torquata,*
- *Lepræa rubra,*
- *Lumbrinereis tenuis,*
- *Nereis limbata,*
- *Pectinaria gouldii.*

Water analyses for this association are shown in Table VII. The table includes the last six items from the preceding table, since the conditions described in those collections are as characteristic of the one as of the other. A discussion of the water analyses for this and the other associations of the eroding shore series is reserved to a later section.

Here this series logically ends so long as the shores remain under erosion with a gradual wearing away of the land to expose more rocks, which would result in the association of the exposed rocks moving gradually forward as erosion proceeds. In case the shore line should remain stationary for sufficient time, the rocks might be worn away to sand and this association might conceivably pass gradually into that of the sand bar which will be considered in the next section. On the other hand if the configuration of the land changes so that currents no longer cause erosion, but deposition sets in, then in the region of the present rock associations one may find sand deposited and the sand bar association might be reached by that route. If the region becomes still more quiet, mud might be deposited and the rock association would then pass to the *Mya* association and with further deposition, pass into the *Uca* or *Melampus* stage. The physical factors correlated with these associations will be given on a later page and anyone interested in completing this series on either of these possibilities, can readily do so.

TABLE VII.

SHOWING OXYGEN CONTENT, TEMPERATURE, pH AND SALINITY OF THE ROCKWEED ASSOCIATION.

Date 1920.	Depth of Sample.	Tide.	Temp.	pH.		O_2.		Time.	Light.	Salinity.	Coll. No.
				Bot.	Sur.	Bot.	Sur.				
					Gansett.						
8/30	2 ft.	Low	23	8.3	8.3	8.56	—	2:45	Bright	—	89
8/30	2.5 ft.	Flowing	22	8.3	—	8.41	—	3:45	Bright	—	91
8/30	3 ft.	Flowing	—	—	8.3	—	—	4:10	Bright	—	93
9/1	6 ft.	Near high	22	8.0	8.0	5.15	—	11:30	Overcast	3.05	96
9/1	1 ft.	Near low	23	8.0	—	5.35	—	2:45	Bright	2.97	103
9/1	2 ft.	Low	21	8.2	—	6.14	—	3:45	Overcast	3.02	104
					S. W. Gutter Creek.						
9/3	2–2.5 ft.	Slack	19	8.0	—	4.75	4.75	2:15	Bright	3.01	115
8/25	1–1.5 ft.	Low to Bay	21	8.0+	8.0+	4.75	5.35	9:10	Bright	2.98	77

2. *Associations of Depositing Shores.*

Notwithstanding the slight amplitude of tides in the Woods Hole region, there are a number of fairly extensive tidal flats which furnish the basis for the associations of the depositing shores. The best of these near the Laboratory is the flat opening off Northwest Gutter just before it enters Buzzards Bay. The majority of collections to be reported were made at this place. Verification studies were carried on in Lackey's Bay, at Gansett, and in Southwest Gutter Creek.

The different animal habitats to be found on the flats grade slowly from one to another so that it becomes impossible to get hard and fast limiting lines. This is true even of the more extensive flats, those at North Falmouth for example, but it is particularly marked in regions such as Gansett where the water deepens fairly rapidly, leaving only narrow zones along the margin. Similar jumbling of habitats is to be found in the salt water creeks of Hadley Harbor and is more characteristic of the Woods Hole coasts than are the more gradual transitions found on the flats proper.

Theoretically the youngest stage in this series is that shown in (*a*) the association of the open water. The conditions in such positions have already been discussed so we shall proceed immediately to the associations of the flats.

b. The Sand Bar Association.—In this region tidal flats are typically separated from the open water by sand bars which may be much exposed at low spring tides, or just covered at low neap tides. The outer part of the bar is relatively pure sand which gradually becomes mixed with humus as one moves back on the flats. The characteristic animals of such an association are given in list 6.

In places practically pure cultures of *Scoloplos fragilis* occur in the sand and if an animal name for the association is desired it might well be called the *Scoloplos fragilis* association.

Where the sand bar extends out to the margin one finds the expected evidences of vertical distribution depending on tide level. At the upper tidal limit congregating under the eel grass occur great numbers of the beach flea, *Orchestia agilis;* burrowing in the sand are the *Talorchestia longicornis* and locally, *Hippa talpoida.*

List 6. Characteristic Animals of the Sand Bar Association.

Cœlenterata:
Hydractinia echinata on shells ocupied by *P. longicarpus*.

Nemertini:
Cerebratulus lacteus,
Micruri leidyi.

Echinoderma:
Leptosynapta inhærans.

Annelida:
Clymenella torquata,
Diopatra cuprea,
Phyllodoce sp.,
Scoloplos fragilis,
Scoloplos robustus.

Arthropoda:
Crangon vulgaris,
Ovalipes ocellatus,
Pagurus longicarpus,
Limulus polyphemus.

Mollusca:
Crepidula convexa, on hermit crab shells,
Crepidula plana, on hermit crab shells,
Ensis directus,
Nassa trivittata,
Natica duplicata, with egg collars.

Chordata:
Dolichoglossus kowalevskyi.

The conditions of the water over this association are shown in Table VIII. The last item in the table was from Blind Gutter Bar. The others are from Northwest Gutter Bar. With the exception of one reading, made in triplicate, the pH ranges from 8.0 to 8.25. In this respect it approaches open water. The observed range is greater than that of the nearby rocks. The salinity is similar to that of the rocks and less than that of the open water. The oxygen content resembles that of exposed rocks where rockweeds are absent. The temperature range is greater than in any other association so far studied and the

Table VIII.

Showing Temperature, Salinity, Oxygen Content and pH of Sea Water over the Sand Bar Association.

Date 1920.	Time.	Depth Water in Ft.	Tide.	Temp. ° C.	pH.	O_2 in c.c. per L.	Light.	Salinity.	Collection Number.
8/14	3:15	1.0	Low	24.5	8.25	—	Dull	3.10	4
8/16	11:30	6.0	High	22.0	8.0	—	Dull	—	13
8/18	2:20	1.5	Ebbing	22.0	8.1	—	Rainy	—	30
	4:00	0.5	Low	21.0	8.1	—	Rainy	—	38
8/23	9:35	0.75	Low	20.0	7.6	3.91	Dull	—	51
8/24	11:15	0.5	Flowing	20.0	8.0	4.63	Bright	2.99	66
9/3	10:30	5.0	Flowing	20.0	8.0	3.95	Sunny	2.99	109
9/3	4:30	1.25	Ebbing	20.0	8.2	4.16	Sunny	3.02	119[1]

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[1] Blind Gutter Bar.

temperature at the upper surface of the sand when fully exposed to the afternoon sun must run much higher than the recorded 24.5° C. observed for the water under these conditions. The pH at Blind Gutter shows the effect of the neighboring vegetation which lies on both sides of this bar.

c. Muddy Sand Association.—The association of muddy sand shades insensibly into the preceding animal community on its outer side. On its inner side it includes the short eel grass (*Zosteria marina*) which extends well out on the sand and then gradually passes into the eel grass and muck association. The most characteristic animal of the association is the sipunculid worm, *Phascolosoma gouldii* and the association might well be called the *Phascolosoma* association. Much of the muddy sand may be exposed at extreme low tide.

This community was most extensively studied at Northwest Gutter, but verification studies were run at other points. Common animals of the association are given in list 7 but the animals shown in the preceding list are also found here.

List 7. Animals Easily Found in the Muddy Sand or *Phascolosoma* Association.

(See also List 6.)

CŒLENTERATA
- *Hydractinia echinata,*
- *Sagartia luciæ.*

ANNELIDA
- *Arabella opalina,*
- *Clymenella torquata,*
- *Drilonereis longa,*
- *Glycera americana,*
- *dibranchiata,*
- *Lumbrinereis tenuis,*
- *Nereis limbata,*
- *Nereis virens,*
- *Pectinaria gouldii,*
- *Phascolosoma gouldii,*
- *Phyllodoce catenula.*

MOLLUSCA
- *Crepidula convexa,*
- *Lacuna vincta,*
- *Lævicardium mortoni*
- *Littorina litorea,*
- *Mya arenaria,*
- *Nassa obsoleta,*
- *Venus mercenaria.*

ARTHROPODA
- *Carcinides mænas,*
- *Heteromysis formosa.*
- *Libinia dubia,*
- *Limulus polyphemus,*
- *Palæmonetes vulgaris,*
- *Virbius zostericola.*

CHORDATA
- *Dolichoglossus.*

The analyses of water over the muddy sand association (Table IX.) show an increase in range in all four factors considered when compared with the conditions found on the sand bar. The majority of these samples are from the edge of the short eel grass. The effect of the eel grass on oxygen content and on pH is noticeable although not so extreme as will be found later. These effects are the expected result of photosynthesis.

Table IX.

Showing Analyses of the Water taken over the Muddy Sand or *Phascolosoma* Association.

Date 1920.	Time.	Depth Water in Ft.	Tide.	Temp. ° C.	pH.		O_2.		Salinity.	Light.	Coll.
					Bot.	Sur.	Bot.	Sur.			
Northwest Gutter.											
8/14	2:10	.25 −.5	Low	25.5	8.45	—	10.39	—	3.10	Dull	1
	4:00	.08	Low	27.0	8.5	—	—	—	—	Dull	1′
	4:05	.5	Flowing	26.0	8.4	—	—	—	—	Dull	6
	4:30	.33	Flowing	27.0	8.45	—	—	—	—	Dull	8
8/16	3:55	1.5	Ebbing	22.0	8.1	—	5.30	—	—	Duller	15
8/18	10:05	4.0	Flowing	22.0	7.9+	8.0−	4.21	—	—	Rainy	25
	2:30	2.0	Ebbing	22.0	8.1	8.1	—	—	—	Rainy	31
	3:45	0.5	Ebbing	—	—	—	4.76	—	—	Rainy	36
8/23	10:00	1.0	Flowing	—	7.8	—	4.2	—	3.04	Dull	52
	1:15	3.0	Flowing	—	8.0−	8.0−	4.75	—	—	Sunny	58
8/24	2:15	2.5	Flowing	21.0	8.0+	8.0+	5.05	5.15	—	Sunny	73
9/3	12:50	5.0	High	21.0[1]	8.1	8.1	4.71	4.63	3.04[1]	Bright	113
Southwest Gutter Creek.											
8/25	10:20	.08	Low	21	7.7	—	—	—	—	Bright	78
	10:20	.67	Low	21	8.0	—	5.25	—	2.97	Bright	78
	12:10	.75	Low	—	8.0	—	5.20	—	—	Bright	80
Gansett.											
8/31	1:45	1.0	Ebbing	23	8.2	—	7.63	—	2.97	Sunny	98
9/1	1:15	3.0	Half	21[2]	8.2	8.0	7.08	6.19	2.96	Bright	101
	4:21	1.0	Flowing	23.5	8.4	—	8.61	—	—	Bright	106

d. Eel Grass and Muck Associations.—The associations of the eel grass and muck lie between the last discussed habitat and a region about a rod from the shore line of the type of flats under discussion. There at a depth of about a foot at low tide, the eel grass ends and the marginal muck association begins. Two major recognizable associations occupy this region: that characterized by the bivalve, *Cumingia tellinoides*, which may be called for convenience the *Cumingia* association and in deeper water and deeper muck, the region characterized by the annelid, *Scoloplos acutus*, which may give its name to this association.

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[1] Same at surface.

[2] 23 at surface.

On account of lack of time no water samples were taken over the *Cumingia grounds*. Enough collecting to yield 25 *Cumingia*, gave the following associates:

Annelida: *Polycirrus eximeus*, *Trophonia affinis*, *Amphitrite ornata*, *Glycera americana* and *Maldane elongata*.

Crustacea: *Panopeus sayi*.

Mollusca: *Tellina tenera*, *Solemia velum*, *Nucula proxima*, *Venus mercenaria*, *Lævicardium mortoni*.

Associated with these one usually finds the burrowing anemone, *Edwardsia elegans;* the swimming annelid, *Podarka obscura;* the shrimps: *Palæmonetes*, *Crangon*, and *Hippolyte;* and the green crab, *Carcinides*.

In both Northwest and Blind Gutters the *Scoloplos acutus* association occupies several acres. At Gansett, the whole center of the bay supports a dense growth of eel grass and from general appearances, I should judge that it would belong to this association but it comes out into shallow water only in a relatively narrow zone and there some of the typical animals, *Thyone briareus*, for example, were not found.

List 8. Showing the Common Animals Found on, among, and at the Roots of Eel Grass in the Center of NW. and Blind Gutter Flats.

A. On Eel Grass.

Coelenterata:
- *Sagartia luciæ* (frequently many).

Bryozoa:
- *Bugula turrita* (much).
- *Schizoporella unicornis* (some).

Gasteropoda:
- *Bittium alternatum* (many).
- *Crepidula convexa* (several).
- *Crepidula fornicata* (few).
- *Littorina rudis* (few).
- *Littorina litorea* (many).
- *Littorina palliata* (few).

Chordata:
- *Molgula manhattensis* (very few).
- *Botryllus schlosseri* (little).

B. Among Eel Grass.

Annelida:
- *Podarka obscura* (frequently abundant).

Crustacea:
- *Virbius zostericola* (very many).

Mollusca:
- *Pecten irradians* (many).

C. In Muck at Roots.

Echinoderma:
- *Thyone briareus* (frequently abundant).

Annelida:
- *Scoloplos acutus* (many).

This association is in the region of tall eel grass which at high tide floats erect, allowing light to penetrate to the bottom. As the tide recedes, the grass falls over and finally at low tide forms dense mats which effectively shade the bottom except in queer open spaces where for some reason the eel grass fails to grow.

The eel grass supports a considerable fauna and flora. Davis (1911) lists 42 species of plants known to occur as epiphytes upon it in the more open situations. The number of animals found in the type of region under discussion is restricted. List 8 shows those found in Northwest and Blind Gutters in this association. The animals listed were collected in about three times the collecting time that gave the *Cumingia* list.

Above the mat of living eel grass at low tide, the temperature runs up as high as 32 degrees C. on a hot afternoon. Just below the matted eel grass, six inches from the water surface the temperature was 27, while at the bottom the thermometer registered 24 degrees. Thus there is a temperature gradient here of eight degrees in 30 inches.

Similar gradients of other environmental factors are present. Thus the oxygen content of the water at the bottom just above the *Thyone* and *S. acutus*, may fall as low as to give a mere trace with the Winkler method, when 24 inches higher, just below the eel grass mat, it was found to be 10.22 c.c. per liter and six inches higher, at the surface, 12.97 c.c. per liter. This makes a total gradient of 12.97 c.c. per liter in 30 inches. The pH gradient was found to be wholly similar: 7.3 at the bottom, 8.5, below and 9.0 above the eel grass matting.[1] These results are the natural concomitants of rapid photosynthesis at the surface of quiet water overlying thick muck.

The greater density was found at the bottom, but when the density was corrected for temperature the determinations showed a higher percentage of salinity at the surface. These and other details are shown in Table X. Particular attention is called to the wide range of variation in the different factors analyzed for this association. In the table, collections 99 and 102 are from

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[1] Under laboratory conditions Atkins ('22) reports that sea water may come to have a pH of 9.77 when jars containing *Ulva* are exposed to sunlight.

TABLE X.

SHOWING THE TEMPERATURE, OXYGEN CONTENT, SALINITY AND pH OF THE WATER OVER THE *Scoloplos acutus* ASSOCIATION.

Date 1920.	Time.	Depth in Ft.	Tide.	Temp. ° C.	pH.		O_2.		Salinity.	Light.	Coll.
					Bot.	Sur.	Bot.	Sur.			
8/14	2:35	.25 –.5	Low	25.5	—	8.45	—	—	—	Dull	2
	3:00	.25 –.5	Low	25.0	8.25	—	—	—	—	Dull	3
	4:00	.25 –.5	Low	26.0	—	8.4	—	—	—	Dull	3′
	3:30	.33	Low	26.5	—	8.45	—	—	—	Dull	5
8/16	1:30	1.25	Ebbing	22.0	8.2	—	—	—	—	Duller	16
	2:10	1.0	Ebbing	23.0	7.9	—	—	—	—	Duller	18
	3:00	0.67	Ebbing	—	7.85	—	—	—	—	Duller	20
8/18	11:00	4.5	High	22.0	8.0	8.0	1.96	—	—	Rain	26
	2:45	1.6	Ebbing	—	8.0	8.2	—	—	—	Bright	32
8/23	10:25	1.0	Flowing	20.0	7.85	8.0	2.37	—	—	Dull	53
	1:30	3.0	Flowing	23.0	—	8.4	7.03	7.81	—	Sunny	58′
	2:00	3.0	Flowing	—	—	8.3	—	—	—	Sunny	59
8/24	9:55	0.75	Low	20.5	7.8 7.7	8.6 – 8.6 –	4.9	8.66	3.02	Sunny	63
	11:30	1.25	Flowing	24.0S	8.3	8.6 –	5.69	9.9	—	Sunny	67
	1:30	2.0	Flowing	25.0S 24.0B	8.2	8.5	4.65	8.91	—	Sunny	72
	2:20	2.5	Flowing	23.5S	8.3 +	8.3	5.54	5.31	—	Sunny	74
	2:50	3.0	Flowing	26.0S	8.35	8.7	9.51	10.7	—	Sunny	75′
9/3	11:50	6.0	High	18.0B 20.0S	8.0	8.1	5.88	5.31	2.98B 3.01 S	Bright	112
8/31	2:30	2.5	Ebbing	22.5B 26.0S	7.8	8.6	3.26	10.6	3.01	Sunny	99
9/1	2:00	3.0	Ebbing	22.0B 22.0S			2.28	7.68	2.99	Bright	102
8/25	2:10	2.5	Flowing	24.0B 32.0S	7.3	9.0	Tr.	12.97	2.88	Sunny	83
	3:05	2.5	Flowing	27.0M	8.5M		10.79M		—	Sunny	83′
9/3	3:40	2.5	Ebbing	20.0B 24.0S	7.8	9.0 –	4.25	10.8	3.02B 3.11 S	Bright	118
8/14	4:15	0.25	Low	26.0	8.0	—	—	—	—	Dull	7
8/18	2:00	3.5	Ebbing	22.0	7.7 +	—	2.43	—	—	Dull	29
8/23	12:00	1.5	Flowing	20.0*b*	7.6	7.9	4.46	4.6	3.02	Bright	56
	1:00	2.1	Flowing	20.0	7.9	7.9	—	—	—	Bright	57
8/24	10:50	0.67	Low	22.0	8.1	8.1	4.95	—	—	Bright	64
9/3	1:00	4.5	High	21.0*c*	8.0	8.1	4.32	4.84	3.01*c*	Bright	114

Collections marked "S" are from the surface; "B," are from the bottom; 'M" are from six inches below the surface. Collections not otherwise labeled are from the bottom.

a. Collection No. 75, although taken after No. 74, was from further inshore and represents an earlier stage in the rising tide.

b. 21 at surface.

c. Same at surface.

Gansett; 83 and 118 are from Blind Gutter Flats. The others are from NW. Gutter Flats.

One open space in the eel grass, similar to those occurring scattered over the flats, except that it is larger and occurs near the margin, is to be found at NW. Gutter where a gravel spit extends from Uncatena Island partially across the creek leading to Hadley Harbor. On the Hadley Harbor side of the spit is a space covering some 2–3 square rods close up in the angle made by the gravel with the shore. The almost bottomless muck is covered over by a thick layer of old eel grass which acts as a very effective matting over the soft ooze below. Such eel grass is very resistant and decays slowly. The main tidal current flows past the edge of this eddy and thus prevents conditions from becoming as extreme as they would otherwise.

In addition to the numerous mud snails (*Nassa obsoleta*), one may find here: a few hermit crabs (*P. longicarpus*) carrying *Hydractinia* and the snail, *Crepidula convexa.* In places the common *Littorina litorea,* almost equals the mud snails in numbers and in other nearby regions only the latter are found. Some *Libinia dubia* are also on the surface while under the matting one finds nothing save a few *Scoloplos fragilis.*

The analytical data collected from this locality is shown in the latter part of Table X, commencing with collection No. 7. In many respects the conditions in this particular location resemble those of the *Thyone* association and seem to present a transition stage between the two associations. This will be discussed in detail in the last section.

e. Marginal Muck (*Thyone*) *Association.*—*Thyone* occurs in the preceding association as well as here, but since they are easily the most conspicuous animals in this environment and there are at least as many of them here as among the eel grass, and they may be absent in typical *S. acutus* conditions, the name is entirely appropriate.

The marginal muck association inhabits the region between the edge of the eel grass and low tide and overlaps both the preceding and the following associations. The eel grass on muddy shores usually begins to grow where the water is from one to two feet deep at low tide. This leaves a space from one to two rods wide on the inner side of Northwest and Blind Gutter Flats, bare of vegetation and with a bottom of soft muck some eight inches or more deep. The *Thyone* bury themselves in this muck, leaving only the tentacles and cloacal opening exposed. They are frequently numerous and may be seen over considerable extent with one or more for every square yard.

Associated with *Thyone briareus*, in such locations are: a few of the worms, *Clymenella;* many *Lumbrinereis tenuis*, and the clam-worm, *Nereis virens*.

Of the crustacea, *Carcinides mœnas*, the green crab, is most conspicuous and may frequently be seen running along the bottom, dodging in and out of the eel grass. The ever-present small hermit crabs, *P. longicarpus*, with their usual commensals, are present in some abundance together with the shrimp, *Crangon vulgaris*. The mud crab, *Panopeus*, is also found.

The molluscs are represented by *Modiolus demissus*, near the edge, where *Mya* and *Venus* also occur. There are also a few strings of *Mytilus edulis* and some rolls of *Crepidula fornicata* to be found. Mud snails, *N. obsoleta*, are also present.

Table XI.

Showing the Results of Analyses of the Water over the *Thyone* Association.

All but the last two items are from NW. Gutter Flats; those are from Blind Gutter Flats.

Date 1920.	Time.	Depth in ft.	Tide.	Temp. ° C.	pH.		Oxygen.		Salinity.	Light.	Coll. No.
					Bot.	Sur.	Bot.	Sur.			
8/16	1:45	1.0	Ebbing	22	7.8	—	4.08	—	—	Dull	17
	2:45	0.67	Ebbing	22	7.8	7.8	—	—	—	Dull	19
8/18	12:30	4.2	Ebbing	22	—	8.0	—	—	—	Dull	27
	3:15	1.0	Ebbing	22	7.4	—	4.45	—	2.22	Rain	34
8/23	11:00	1.0	Flowing	20	7.9	7.9	4.53	—	3.01	Dull	54
	11:30	1.5	Flowing	20.5	—	—	—	—	3.01	Dull	55
	2:00	3.0	Flowing	20.5	8.2	8.2	6.20	8.37	—	Bright	59
5/24	9:15	1.0	Ebbing	21	7.8	7.8	3.96	—	3.04	Bright	62
	12:30	1.0	Flowing	24	8.0	—	—	—	—	Bright	69
	3:15	3.0	Flowing	26	—	8.5	—	—	—	Bright	76
9/3	11:30	4.5	High	17B 20S	7.4	8.2	3.23	5.51	2.88B 3.02 S	Bright	110
8/25	3:45	1.0	Flowing	28	8.2	—	6.79	—	3.10	Bright	84
9/3	3:20	1.6	Ebbing	22	8.0	—	5.31	—	3.08	Bright	117

B, collection from bottom; S, collection from surface.

Except for salinity, the water over the marginal muck association shows much less range in variation than in the *S. acutus* association. Thus the recorded temperature range is 11° C. against 15°; the pH range, 0.8 *vs.* 1.7; the oxygen, 5.24 c.c. per liter against 12.97. These are the natural results of the lack of green plants in this association. On the other hand a careful (laboratory) determination of salinity, made just after a heavy shower that was one of a series running through a whole morning, showed the effects of fresh water drainage from a nearby swale by recording a salinity of 2.22 per cent. It is probable that this lessened density extended into typical *Scoloplos acutus* territory, though in a less marked form, but no tests have been made there under these conditions. Dilution after a really heavy rain would be much greater. The results from analyses of water over the *Thyone* beds are shown in Table XI.

f. The Intertidal Associations.—In this area the flats extend back to old shore lines where the rocks characteristic of shores are being gradually covered by mud accumulations; these are the typical mud flats which I have been describing. These margins, rising more or less steeply from the water, are the home of the intertidal associations. Most obviously there are two of these: that of the animals near the low tide level, and that near high tide. The former is dominated by *Mya* and is best understood if called the marginal *Mya* association; the latter association depends on location for its typical animals. That most studied has been the *Melampus* association.

(1) *The Mya Association.*—In the localities studied for this report, the *Mya* association is best represented along the west shore of Northwest Gutter Flats. The rocks there are an extension of those of the exposed rock association, but since they are located in the back part of the flats, mud has been deposited around them until in many places they are almost entirely covered up to the mid-tidal region. Here from well below low tide to about mid-tide the siphons of small *Mya* [1] are so numerous as to appear like stippled spots against the mud background. In the lower part, the larger siphons of *Venus* give variety to the stippling. If one try to dig up the clams, he encounters the buried rocks within a few inches of surface.

[1] Mr. G. M. Gray informs me that he has never taken *Mya* in pure muck.

Other animals to be found here are: *Natica duplicata, Nereis virens, Polycirrus eximeus,* burrowing in the mud; *Nassa obsoleta* and the hermit crab, *P. longicarpus,* with its commensals, *Hydractinia* and *Crepidula convexa,* on the bottom, retreating as the tide ebbs; and *Ostræa virginica* growing occasionally on the rocks.

TABLE XII.

SHOWING THE RESULTS OF ANALYSES OF THE WATER OVER THE *Mya* ASSOCIATION.

Date 1920.	Depth in Inches.	Tide.	Temp. ° C.	pH.	Oxygen c.c. per Liter.	Light.	Salinity %.	Time.	Collection Number.
8/16	1	Ebbing	28.0	7.5	—	Dull	—	3:15	21
8/18	1	Ebbing	22.0	7.1	—	Dull	2.22	3:00	23
8/23	1	Flowing	20.0	7.9	—	Dull	—	11:00	54
	1	Flowing	20.5	8.2 +	—	Bright	3.01 3.01	2:30	60
8/24	1	Ebbing	21.5	7.8	3.72	Bright	—	9:00	61
	1	Flowing	27.0	7.6	—	Bright	3.04	12:15	68
	1	Flowing	28.0	8.0	—	Bright	3.10	1:25	70
9/3	36	High	19.0	7.8	4.86	Bright	3.04	11:45	111

The temperature of the water in this association runs much higher than on the rocks; 28 degrees C. was recorded. The exposed mud at low tide must run still higher. Few oxygen tests were made here, but they showed, as was expected, that there is a good supply of oxygen in the water. In the upper reaches, at least, the water does not cover the mud long enough to lose much of its supply which came in with the high tide. Under low tide conditions the mud must contain almost no oxygen at all. The pH may be markedly lower than on the other associations, correlated with the fact that the muck is acid. The observed salinity is the same as for the marginal muck association but it probably runs lower on occasion, from the drainage after rains.

TABLE XIII.

SHOWING THE TEMPERATURE, OXYGEN TENSION, AND pH OF THE *Melampus* ASSOCIATION.

Date 1920.	Depth in Inches.	Tide.	Temp. ° C.	pH.	Oxygen c.c. per Liter.	Light.	Salinity.	Time.	Coll. No.
8/16	1	Ebbing	23	7.4	1.96	Dull	—	12:00	14
8/18	1	Ebbing	22	7.2	1.81	Raining	—	1:30	28

(2) *High Mud Shores* (*Melampus*) *Association.*—The last type of habitat in the series comes with the further deposition of organic mud. Under these conditions the *Mya* association would gradually be raised above low tide level and finally above mid tide level. Marsh grasses would come in and in place of the association dominated by *Mya* there would be one dominated in places by the fiddler crabs (*Uca*) and in others by the snail *Melampus lineatus*. The situations studied have had more of the latter than the former.

The *Melampus* grounds are well illustrated in the Uncatena shore of Northwest Gutter from near the bridge to the gravel spit marking the eastern side of the passage to the Bay and at various places along the other gutter creeks, especially near the rockweed association already located.

The substratum is a peaty mixture of muck and roots which is covered only at high tide and then by only a few inches to a foot or so of water. In the lower parts the mussel *Modiolus demissus* is present sometimes in abundance. At the upper side the land insects, such as the carabid beetles, are plentiful, while Thysanura are abundant over much of the region at low tide. The ground is moist at all times on account of the spongy character of the substratum and the protection furnished by marsh grasses.

Too few analyses of water of this region have been made but the low pH and accompanying low oxygen content of the water as it leaves the flat when the tide ebbs, is significant. When this water flows over the *Melampus* grounds, it would have a pH of about 8.0 and an oxygen tension of some 4.00 c.c. per L. The locality most studied would be covered with water about six hours of the twenty-four. The salinity must vary from about 3.00 per cent. at high tide to practically fresh water when low tide coincides with a heavy rain.

IV. SUMMARY OF DATA.

There are two types of data which one desires concerning the environmental complex of animals: First, what are the average conditions during the period when the divisions between habitats are most pronounced? And second, what extremes are to be found within a given association? The available data on these points for the months of August and early September, will be presented by tables for the two series studied.

a. Rocky Eroding Shores.—The average conditions in the eroding shore series when conditions are most acute, are shown in Table XIV. This series is composed of the associations of the open water, wharf pilings, exposed rocks, and rockweeds growing on protected rocks. Data from the intertidal associations at the back of mud flats are added for comparisons.

TABLE XIV.

ERODING SHORE SERIES.

Showing average salinity, oxygen content, temperature, and pH of different animal associations as found in collections from the bottom at low tide except for salinity where low and mid-tide data are averaged. The last two associations are added for comparative purposes.

Ordinal ranking is based upon variation from conditions known to occur in open water.

Association.	Salinity.			O_2 c.c. per L.			Temperature.			pH.			Sum of Ranks.
	No. Rec.	Ave. ‰.	Rank.	No. Rec.	Ave.	Rank.	No. Rec.	Ave. ° C.	Rank.	No. Rec.	Ave.	Rank.	
Open	12	3.12	1	12	5.17	1	10	21.1	2	15	8.0	1.5	5.5
Wharf pilings	4	3.05	2	12	4.72	2	11	20.0	1	13	8.0	1.5	6.5
Exposed rocks	6	3.02	3	8	5.91	3	11	21.4	3	9	8.16	4	13
Rockweed	4	2.99	4	6	6.33	4	6	21.5	4	6	8.13	3	15
Mya	5	2.88	5	1	3.72	5	7	24.0	6	7	7.7	5	21
Melampus	0	—	6[1]	2	1.88	6	2	22.5	5	2	7.3	6	23

The average salinity at low and mid-tides decrease in the order given. The average low tide temperatures increase in the order given from the wharf pilings but these, contrary to expectation, are lower than the open water. The average oxygen tension varies from that found in the open water by amounts which likewise arrange the series in the order given. Thus the wharf pilings gave an average of 4.72 c.c. of oxygen per liter which is 0.49 less than the average found in open water; while the exposed rock association with an observed average of 5.1 c.c. per liter is 0.74 c.c. from the open water conditions.

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[1] Must range from almost fresh water during rain at low tide to about 3.00 at high tide.

The pH averages the same in the wharf pilings and open water associations. The rockweed association comes next with the association of exposed rocks very near it. The pH values from the exposed rock association is higher than would be expected for the entire coast because only one of the three places in which it was studied was free from the influence of nearby submerged vegetation. Under entirely typical conditions the average pH in this series should likewise arrange the series in the same order as the other factors measured.

The rankings given in the table are based upon the extent of variation from conditions known to be characteristic of open water. The sum of the ordinal ranks, a poor enough method of averaging, shows a gradual increase corresponding to the ecological age of the associations.

The range of these four factors (Table XV.) tends to increase with the age of the association. This is not true of each factor taken separately, nor is the increase in total amounts in regular order, in that the rockweed association on this basis precedes the exposed rock association, when it would be expected to follow it.

In addition to the absolute range the data from these extremes show another set of relations not given in averages, that is the relative position of the extremes. In salinity of the open water the lowest record is fairly high and, while the upper extreme of salinity remains approximately constant, there is a general fall in the lower limit as one passes from open water to the older associations.

In the temperature series the characteristic change is in the matter of the greater maximum in the older associations. With pH, the young associations have practically no change, the older ones have an increased upper limit due to the action of plants, while the oldest ones show a decided decrease in the minimum on account of the acid-giving muck which is deposited in them.

A combination of the rankings based on the departure of average low tide conditions from those known to occur in open water and on amount of variation between observed extremes, places the associations in order of their ecological age, with the exception of the rocks and rockweed associations which are placed together. If in place of an index figure based on amount of variation of extremes one substitutes the more complex index shown in Table XV., which combines the amount of variation with the location of extremes, the series is arranged by the data given here exactly in order of ecological age.

TABLE XV.

ERODING SHORE SERIES.

Showing range of variations of temperature, oxygen content, salinity, and pH at the stratum most studied in each animal association; all tide stages considered. The last two associations are added for comparative purposes.

Ordinal ranking is based on amount of variation and relative location of extremes.

Association.	Salinity in Per Cent.			Oxygen in c.c. Liter.			Temperature in Degrees C.			pH.			Ranking.		
	Limits.	Range.	Ordinal Rank.	Limits.	Range.	Ordinal Rank.	Limits.	Range.	Ordinal Rank.	Limits.	Range.	Ordinal Rank.	Sum of Ranks.	Combined Ranking.	Final Ranking.
Open	3.02 3.14	0.12	1	3.93 5.43	1.50	1	20 22	2.0	1.5	8.0 − 8.0 +	0.01	2	5.5	11	1
Wharf pilings	2.97 3.13	0.16	2	4.06 6.00	1.94	2	19 21	2.0	1.5	8.0 8.0	0.0	1	6.5	13	2
Exposed rocks	2.94 3.04	0.10	3	3.62 8.56	4.94	4	20 23	3.0	3	8.0 8.3	0.3	3.5	13.5	26.5	3
Rockweed	2.97 3.02	0.5	4	4.75 8.56	3.81	3	19 23	4.0	4	8.0 8.3	0.3	3.5	14.5	29.5	4
Mya	2.22 3.10	0.88	5	0.0[1] 4.86	4.86	4.5	19 28	9.0	5.5	7.1 8.2	1.1	5.5	20.5	41.5	5
Melampus	slight to high tide cond.[2]		6	? high tide		4.5	high tide ?		5.5	7.2 high tide		5.5	21.5	44.5	6

b. The Depositing Shore Series.—The flats series intergrades more closely than the different associations of the rocks. The conditions characteristic of each association are most marked at low tide and these are summarized as averaged data in Table XVI. As might be expected the average salinity at low tide decreases regularly as one goes back on the flats. But this is the only factor considered that shows such a regular relationship. With the others the conditions over the sand bar approach those

[1] Estimated between tides.

[2] Estimated for purposes of comparison.

"Combined ranking" refers to the rankings given in Tables XIV. and XV. The final ranking is based on the preceding column.

of the open water most closely and the conditions in the intertidal associations at the back of the flats are normally most extreme, but in the middle regions the plant growth, mainly eel grass, affects conditions so that the relationship is irregular. In the summation of ordinal rankings the effect of this confusion is shown.

Table XVI.

Flat Series.

Showing the average salinity, oxygen content, temperature and pH of different animal associations of the flats as found in collections from the bottom, open water excepted, at low tide, except for salinity where both low and mid tides are averaged. Ordinal ranking is based upon variation from conditions prevailing in open water.

Associations.	Salinity.			Oxygen in c.c. per L.			Temperature.			pH.			Ranking.
	No. Collections.	Average.	Rank.	No. Coll.	Average.	Rank.	No. Coll.	Average.	Rank.	No. Coll.	Average.	Rank.	Sum of Ranks.
Open	13	3.12	1	12	5.17	1	10	21.1	1	15	8.0	1	4
Scoloplos fragilis	3	3.04	2	3	4.23	3	6	21.3	2	6	8.04	2	9
Phascolosoma	6	3.00	3	4	6.15	4	12	23.3	6	13	8.15	5	18
Scoloplos acutus	6	2.97	4	9	3.88	5	15	23.1	5	17	7.95	3	17
Thyone	6	2.91	5	6	4.85	2	9	22	3	8	7.9	4	14
Mya	5	2.88	6	1	3.72	6	7	24	7	7	7.7	6	25
Melampus	0	—	7[1]	2	1.88	7	2	22.5	4	2	7.3	7	25

The amount of range, Table XVII., also increases steadily as one passes back on the flats only in the case of the salinity measurements. The oxygen content and pH begin to increase in observed range as soon as the eel grass is encountered in the muddy sand association and reach their maximum range in the tall eel grass of the *Scoloplos acutus* grounds. The water temperatures also increase in range but become greatest in the low water of the marginal muck association. The surface in the older associations undoubtedly becomes much warmer when exposed to air and this must be particularly marked in the *Melampus* association where the surface of the ground may be exposed to the full glare of the afternoon sun with almost no protection from the scant growth of marsh grasses.

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[1] See note at bottom of Table XV.

TABLE XVII.

FLAT SERIES.

Showing range of variation of temperature, oxygen content, salinity and pH near the surface in the open water and at or near the bottom in other associations. All tide stages considered. Ordinal ranking is based upon amount of variation and relative location of extremes.

Association.	Salinity in %.			Oxygen in c.c. per L.			Temperature.			pH.		Ranking.			
	Limits.	Range.	Rank.	Limits.	Range.	Rank.	Limits.	Range.	Rank.	Limits.	Range.	Rank.	Sum of Ranks.	Combined Ranking.	Final Ranking.
Open	3.02 3.14	0.12	1	3.93 5.43	1.50	1	20 22	2.0	1	8.0 − 8.0 +	0.1	1	4	8	1
Scoloplos fragilis	2.99 3.10	0.11	2	3.91 4.63	0.72	2	20 24.5	4.5	2	7.6 8.25	0.65	2	8	17	2
Phascolosoma	2.97 3.10	0.13	3	4.21 10.39	6.18	4	21 27	6.0	3	7.7 8.5	0.81	3	13	31	3
Scoloplos acutus	2.88 3.02	0.14	4	trace 9.31	9.50	5	18 26.5	8.5	4	7.3 8.45	1.15	4	17	34	5
Thyone	2.22 3.10	0.88	5.5	3.23 5.31	2.08	3	17 28	11.0	5	7.4 8.2	0.8	5	18.5	32.5	4
Mya	2.22 3.10	0.88	5.5	0.0[1] 4.86	4.86	6.5	19 28	9.0[2]	6	7.1 8.2	1.1	6	24	49	6
Melampus	slight to high tide cond.		7	? high tide		6.5	high tide ?		7	7.2 high tide		7	27.5	52.5	7

The relative position of the extremes in salinity is much the same as in the eroding shore series. At high tide the salinity is practically constant throughout, but at low tide the lower limit decreases regularly with distance from the open water. The relations of oxygen content, pH, and temperature at the bottom are exactly similar to those of the preceding series except that they are more striking.

The ranking in this series, as in Table XV, is based upon the amount of range combined with the relative location of the extremes. On this basis salinity, temperature, and pH arrange the series in order of the ecological age of the different associations. On the basis of oxygen content, the marginal muck association is much younger than is expected. The combination of these rankings arranges these associations in order of their age but when combined with the rankings from average conditions shown in the preceding table, the marginal muck (*Thyone*) association is placed slightly earlier in the series than it belongs. Further data would probably adjust this arrangement.

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[1] See note at bottom of Table XV.

[2] Water temperatures only considered.

At the surface of this series, the range of oxygen, pH and temperature is greatest in the *Scoloplos acutus* region. The mat of eel grass just at the surface at low tide allows a surface layer of very warm water to be found in the bright sunlight where it is supersaturated with oxygen and has a correspondingly high pH. The *L. litorea*, *S. luciæ*, *Bugula*, *C. convexa*, *B. alternatum*, *Molgula*, and *Botryllus*, which occupy this region must be very resistant to these extreme conditions. Under the most pronounced conditions few animals capable of moving are found at the surface.

The differences on the flats level off at high tide. The tide appears to come to the back of the flats over the surface of the more stagnant water which has remained behind during low tide, bringing lower temperature, higher salinity, lower oxygen content and lower pH. Thus two collections from the back of the flats in 4.5 feet of water on a flowing tide showed a specific gravity of 1.018 at the bottom and 1.022 at the surface. Obviously such conditions do not prevail long and by diffusion the gradients disappear. In the long eel grass (*S. acutus* association), the vertical gradient at low tide in one foot of water was found to be from 3.37 c.c. oxygen per liter to 10.39 at the surface. Three hours later in the same place in three feet of water of the new tide the gradient was from 7.03 at the bottom to 8.3 at the surface. Similarly a pH gradient of from 7.7 at the bottom and 8.7 at the surface became one of 8.0 to 8.1.

The condition of the water over the bottom of the flats at high tide tends to become uniform throughout all the associations. Thus at NW. Gutter Flats, collections made under typical high tide conditions showed a range of 0.1 in pH over the flats while at low tide the range at similar stations was over 1.2.

V. Discussion.

Sumner (1908) in discussing the study of the distribution of bottom living animals in the Woods Hole region considered character of bottom, depth, temperature, salinity, purity of water and currents as the important factors in determining distribution. The first of these factors, character of bottom, is characteristically different in the associations of eroding and depositing shores. The former is characterized by the presence of firm places for attachment and difficult burrowing conditions; the latter, by the converse of these conditions. The substratum also serves largely in distinguishing between the different associations of each series.

The influence of depth as a factor in animal distribution in the region covered by these studies is not due to depth as such but to depth as assuring a constant supply of water. This is shown in the rockweed association where animals normally found below low tide level on the wharf pilings may live well above it when protected from drying and from high temperatures by the mat of rockweeds.

Temperature serves as a limiting factor for these associations, during the season of the year studied, in the tide pools and more particularly on the flats. There in the *Scoloplos acutus* association above the dense eel grass mats at low tide the high temperature (32° C.) that may be reached must serve to kill off the more sensitive sessile animals as it drives the motile ones below the surface layer to the cooler water in the shade of the eel grass. The temperature to which animals may be exposed at low tide increases as one leaves open water conditions in both series. The effect of this high summer temperature of the flats as a factor limiting the geographic distribution in this region will be discussed in Study IV.[1] of this series.

In the associations studied, the salinity regularly increased as one approached open water conditions. The low salinity on the back part of the flats particularly in the *Melampus*, *Mya* and *Thyone* associations must serve as a limiting factor. These are subjected to such extreme ranges of salinity following heavy rains, particularly if these rains come at low tide and where there is some considerable surface drainage, that sensitive animals or animals in a sensitive stage in their life history must needs be killed or driven off.

In the locations studied, there was no contamination from sewage wastes, so this possible factor in distribution may be dismissed.

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[1] In press in *Ecology*.

The effects of tidal currents are well illustrated in the gutters and creeks of Hadley Harbor. These protected channels, supplied with constantly changing water which differs from open water only by the effect of coming over large tracts of submerged vegetation, support a wholly different animal life from that present where such currents do not enter. These locations are in the exposed rock or the rock-rockweed stage of development, in place of the *Mya* or *Melampus* stage which they would occupy if the currents were absent. In addition to these scouring effects of tidal currents, they have the well known function of oxygen and food carriers. They also eliminate the depth gradients in oxygen and pH found commonly in the stagnant water of the older associations.

The relation of oxygen and pH of the sea water to animal distribution has received no attention in the Woods Hole region. Both depend (1) on the supply of offshore water, (2) the amount of photosynthesis being carried on nearby, and (3) the character of the bottom. In the open water these factors depend upon currents and the proximity to vegetation. In the presence of vegetation oxygen is given off and the pH is increased. Muck absorbs oxygen and lowers the pH while sand has no effect on either unless it has been laid bare, when it, as well as rocks under similar conditions, decreases the pH without affecting the oxygen supply. Wave action has the converse effect.

From examining data from such regions as the rockweed or eel grass one might be tempted to generalize and say that as the oxygen increases the pH likewise increases and *vice versa*. Such a conclusion at best holds only in regions of abundant vegetation or of muck, and there the pH changes lag behind changes in oxygen concentration.

If the invertebrates are as sensitive to pH variations as Powers found herring to be, this correlation of high hydrogen ion concentration with the low oxygen regions of the muck must serve to keep free moving animals out of such conditions. Such action, combined with its greater regularity of distribution and slower fluctuations, makes the pH of the water more important in such studies as the present than is the distribution of oxygen.

Both factors, however, may range widely within a given association. Take for example the sensitive *Amarœcium* association which flourishes on wharf pilings or on exposed rocks. At the mouth of Northwest Gutter, where the pH stays about 8.0 and the oxygen ranges from 3.72 to 4.95 c.c. per L., the rocks support a typical rock-*Amarœcium* association. Yet the same association, fully as rich in species, occurs at Gansett on similar rocks, but with much plant life all about and with the pH varying from 8.0 to 8.3 and the oxygen from 5.15 (and probably lower) to 8.56 c.c. per L. It will be noted that the range of conditions either at Northwest Gutter or at Gansett is lower than when the two are combined, so that, while the association can exist in these limits, it is not subjected to these extremes in one locality. The total range in both temperature and salinity is likewise greater when the two locations are considered together than when either is taken singly.

It is probable that a collection of data concerning the conditions under which the different sensitive animals live in all their different localities would, when thrown together, indicate that they could stand widely differing concentrations of all the water factors considered; when, as a matter of fact, they are exposed to relatively slight changes in the location in which they do live.

With less sensitive animals the association limits as set out in this paper mean nothing. *Pagurus longicarpus* apparently roams at will in all of those of the flats and occurs among those of the rocks, carrying with him, willy-nilly, his commensals. The mud snail, *Nassa obsoleta,* is at present found among all the associations, from the clean sand to the inter-tidal associations, and, according to Dimon, originally dominated the sand also before being driven off by *Littorina litorea.* In part it is able to do this because it is a resistant animal and in part on account of the fact that it probably becomes accustomed to conditions in a given locality and tends to keep within them. In this regard, one can but express the wish for more studies like that of Dimon. With a series of such studies at hand one could draw definite conclusions where now, so far as individuals are concerned, he is limited in large part to theorizing.

There is so much work necessary in making an ecological survey that there is always a lively interest in single factor indices of associations, and at the beginning of the present studies I rather expected to find such an index in the pH relations. While a combination of the average pH, the extent of range and the relative position of the extremes does allow one to place these associations in their natural order with considerable exactness, and while such data is very suggestive it does not classify these associations with the precision necessary for a successful single factor index. This is emphasized in the rock series of associations, Tables XIV. and XV.

The use of the oxygen content of the water is out of the question as such an index of an association. Temperature is an aid but needs confirmation. Salinity, whether average or range, considered with position of extremes, does arrange the different associations in their logical order, and so qualifies for the recommendation that was predicted for pH, as the best single index when water conditions alone are considered. The readily determined factors of salinity, temperature and pH taken together give a much stronger index than any one of them alone.

If, however, on account of urgent haste, I should be forced to make use of a single criterion to divide the communities of the Woods Hole littoral, I should depend more on observation of the character of the sea bottom than on any other one factor.[1] This, the most obvious, the longest used, is still the least treacherous single factor index of littoral distribution in this region. It should be used with discretion since a rock well back on the flats supports a different set of animals from one on an exposed point, but the corrections are more obvious and more easily applied.

All the data collected in the present investigation agree in supporting the common sense conclusion that animal associations in a region such as this under consideration are not normally limited by any one factor, but by the interaction of several, and when feasible all these should be analyzed and recorded.

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[1] Shelford ('14) in writing of the suitability of water for fishes concluded that the amount of clean bottom, the amount of carbon dioxide and the amount of hydrogen sulfide, taken together serve as an index of availability of bays and enclosures of the seas for fish life. Longley ('22) in studying the local distribution of Tortugas fishes concludes that the local distribution of many species is determined by the character of the bottom. This holds particularly for what he calls the "sand-patch" association.

The ecological age of the different associations has been repeatedly mentioned. This idea clearly and repeatedly stated by such workers as Cowles, Shelford and Adams, is apparently not yet fully understood. Briefly, it means that the *Melampus* association as it exists at present is an old association which has passed successively through the other stages standing before it in the series. Thus at one time the spot on which a *Melampus* association is now located was bare sand, which, as it became finely ground and somewhat packed, began to support a *Scoloplos fragilis* colony much as is found on Blind Gutter Bar at the present time. With accumulation of the organic products of these animals in the absence of a scouring current, other animals came in until the *Scoloplos fragilis* association began to resemble a *Phascolosoma* association. As more muck was deposited the older associations were passed one by one until the present old *Melampus* association resulted. With further deposition the land will be raised above tidal level and the *Melampus* association will gradually give way to strictly land animals. In this region the muck that accumulates is almost entirely of organic origin (cf. Survey, p. 32) so that the animals themselves have played a considerable part in causing ecological succession to take place.

Transitional stages between the different associations are abundant. A particularly noticeable one was studied that belongs between the *Scoloplos acutus* and *Thyone* associations. This is located near the gravel spit at the Uncatena side of the Northwest Gutter passage. There the *Scoloplos acutus* occur in the muck, but *Thyone* are absent as yet, although they are found nearby in greater numbers each year. Of the eight comparisons that have been made in the summary tables, four would place this location with the *Thyone* association, three place it as younger than *Phascolosoma* association, and the other with *Scoloplos acutus* where it belongs according to its animal inhabitants, and according to the average of these physical factors.

The wharf pilings present a specialized *Amarœcium* type of association that is obviously younger than the rock-*Amarœcium* community, although closely related to it both in animals present and in physical conditions. It represents more nearly the type of habitat that might be found where large rocks extend up out of deep inshore water. Being a man-made habitat it is not, strictly speaking, a part of the rock series and is included in that series because it does give approximately the same condition that would be found on the rocky pillars just mentioned.

The succession of forms in this association can readily be studied. Glass slides placed under wharfs furnish a convenient method of finding the first pioneers to be expected. Observation of the growth of communities on new non-creosoted pilings and comparison with those on middle aged and old pilings would give the whole story for the wharf pilings, since they will of course never become a *Mya* association, as a rock-*Amarœcium* association may, or in the same way that its rock pillar prototype might.

In addition to such a study as this and to the studies of the ecology of individual species, in order to describe completely the littoral ecology of this region, studies should be made in the late autumn, early spring and in late spring or early summer. This last is particularly needed to round out our knowledge of the physical and faunistic conditions in these associations during the spring reproductive period.

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CONTENTS

Hoadley, Leigh. *Certain Effects of the Salts of the Heavy Metals on the Fertilization Reaction in Arbacia punctulata* 255

Watt, James C. *The Behavior of Calcium Phosphate and Calcium Carbonate (Bone Salts) Precipitated in Various Media, with Applications to Bone Formation* . . 280

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BIOLOGICAL BULLETIN

CERTAIN EFFECTS OF THE SALTS OF THE HEAVY METALS ON THE FERTILIZATION REACTION IN *ARBACIA PUNCTULATA*.

LEIGH HOADLEY,

THE HULL ZOÖLOGICAL LABORATORY, UNIVERSITY OF CHICAGO.

I. Introduction. . . . 255
II. Normal fertilization reaction in *Arbacia*. . . . 258
III. Methods. . . . 258
IV. Experiments. . . . 260
  a. Data on various metals. . . . 260
  b. Comparative tests. . . . 271
  c. Summary. . . . 274
V. Discussion. . . . 275
VI. Conclusions. . . . 277

I. INTRODUCTION.

At the suggestion of Prof. F. R. Lillie in the spring of 1921, I began, the following summer, a study of the effects of the heavy metals on the fertilization reaction in *Arbacia punctulata.* I desire here to express my appreciation for his many helpful suggestions and his aid in the interpretation of the results.

Lillie ('21) found that copper salts completely prevent the elevation of fertilization membranes in *Arbacia* even at such a great dilution as 1 : 500,000, though the concentration of copper chloride required for the suppression of cleavage is 1 : 62,500. It is the purpose of the present set of tests to establish the effects of a series of metallic salt solutions on the fertilization reaction and by this means to throw more light on the initial events of this reaction. The work was done during the summers of 1921 and 1922 at the Marine Biological Laboratory at Woods Hole, Massachusetts.

The experiments brought out very clearly that there is a sharp distinction between the initial and the subsequent events of the fertilization reaction. As soon as the initial part of the reaction is completed, *i.e.*, as soon as the membrane has raised, the metallic concentration which has formerly been inhibitory in its action ceases to be wholly effective and some of the eggs may develop as far as the motile free-swimming forms in the solution. In the following pages, such a concentration, which inhibits the elevation of membranes, when eggs are inseminated in it, will be termed "membrane-inhibitory." Such inhibition of membrane elevation is complete. If, however, the concentration of the salt is greatly increased, a concentration may be reached at which cleavage in turn is completely inhibited, but here again, the time factor is important. For example, if a given concentration is toxic to cleavage for a set of eggs transferred to it five minutes after insemination in normal sea water, it is said to be "cleavage-toxic." It may allow 40 per cent. to 50 per cent. cleavage if eggs are transferred to it 20 minutes after such an insemination, or even 95 per cent. if they are transferred after a 30-minute interval. In such a case, second cleavage will be completely inhibited. When cleavage is used as a criterion of toxicity, the period of exposure to the solution will vary according to the length of time elapsing between insemination and transfer. If, when eggs are transferred to the solution five minutes after insemination, first cleavage is inhibited, then it follows that eggs transferred at a later time will not perform second cleavage, though they may cleave once.

McGuigan ('04), v. Euler and Swanberg ('21), Olsson ('21), and others have investigated the effects of the salts of certain of the heavy metals on enzyme action. Mathews ('04), working with *Fundulus*, compares the physiological action of the heavy metals with their solution tensions. It was with the same idea that McGuigan worked on the ferments. He found that, generally speaking, a low solution tension was indicative of great toxicity. There were, however, certain exceptions to the series. v. Euler and Swanberg attribute the poisonous effect of the salts to their property of binding certain groups of the enzyme molecule and thus making it ineffective.

In the ferment experiments just cited as well as in the experiments made by Mathews on *Fundulus*, the experiments allowed the use of distilled water in the solutions, and in consequence, the dissociation of the salts could be accurately measured and precipitation did not trouble the investigators. In the work with *Arbacia*, on the other hand, it was necessary to use sea-water for all of the final solutions though the stock solutions were made in distilled water. The composition of the sea-water is of such a nature that in many cases there is a precipitation and in no case could the ionization of the salt be accurately determined. The sea-water, as it comes from the tap in the laboratory, has a p.H. of 8.0 and I may say here, that in no case did the concentration of the metallic salt necessary to inhibit membrane formation have a p.H. below 7.0. In some of the solutions there was a visible precipitate, as was most evident in the cases of Zinc, Lanthanum, and Lead. In such solutions, the balance of the salts must be disturbed. Thus, in the case of the alkaline earth metals, and silver, tin, manganese, chromium, and iron, the precipitate was so heavy that after a few preliminary experiments, their use was abandoned. The precipitation of zinc, lanthanum, and lead was not great enough to hide the effect of the salts on the elevation of the membranes but when the concentration was increased for the investigation of cleavage-toxicity, the precipitation was so heavy that it was deemed unwise to try to determine that point for them.

When the series was completed, it was found that the work included tests made with the following metals: Gold, copper, zinc, lanthanum, aluminum, platinum, lead, nickel, cadmium, cobalt, and mercury. The results of the experiments show a similar behav or of the metals, save mercury, and those described by Lillie ('21) for the chloride of copper. The inhibiting concentration varies for the different metals.

The behavior of mercuric salts is peculiar and unexplainable at present. Its solutions seem to favor membrane elevation up to a concentration at which the sperm are immediately paralyzed, while at a much greater dilution, cleavage is completely inhibited. This will be treated in more detail in the following pages.

Rhubidium and cesium were found to be indifferent up to the isotonic concentration and will not be treated further.

II. The Normal Fertilization Reaction in *Arbacia Punctulata.*

In order to understand the later discussion, it will be wise here to summarize the phenomena of the normal fertilization reaction in *Arbacia.* These were described in great detail by Lillie ('14), though some of the events had been incompletely described by von Dungern ('02). Fol ('79) described the penetration of the spermatozoön and the elevation of the fertilization membrane in a classical monograph.

Before the spermatozoön comes into contact with the egg itself it is activated and directed toward the egg as described by Lillie ('13). The egg, from the time of rupture of the germinal vesicle up to the time of fertilization, or, when fertilization is barred, up to the time of cytolysis, produces an iso-agglutinin (Lillie '13) or fertilizin (Lillie '14) which produces a reversible agglutination of the spermatozoa to the egg. Between the time of the agglutination of the spermatozoa to the egg and the beginning of the fertilization reaction, there is a latent period which is very short but variable in *Arbacia.* This is followed by a very rapid initial reaction which produces a sterilization of the egg against other spermatozoa, described by Just ('19) as "a wave of negativity." The penetration of the spermotozoön occurs immediately, followed by the elevation of the membrane. In a given lot of fresh eggs, the time elapsing before membrane elevation is very variable. At 19.5° C. some eggs appear with membranes fully formed in from 15 to 30 seconds while others require much longer. Elevation of the membrane is too rapid for observation in *Arbacia* and it seems probable that the variable is due to the latent period. With stale gametes the process may extend over five minutes or more and is often accompanied by polyspermy.

III. Methods.

The experiments described in the following pages are very simple. Eggs were removed from good ripe females by making a circumferential cut around the animal and removing the gonads intact to about 150 c.c. of sea water in a finger bowl. The ovaries were then cut a number of times and the ova allowed to stream forth. In about five minutes, the suspension was strained through cheese cloth to remove the pieces of the ovaries. The eggs were then allowed to settle. When they had settled, the supernatant liquid was poured off and the dish again filled with fresh sea water. This process was repeated three times so that all traces of the coelomic fluid and the tissue juices were practically eliminated. Eggs were not used unless the normally fertilized control showed above 95 per cent. membrane formation and a good viability through the first cleavage.

Males were cut in the same way and placed in a Syracuse glass with the genital pores down. The sperm flows from the pores in a creamy mass, which, when mixed with sea water in the proportion of one drop of dry sperm to 25 c.c. of sea water gives a grayish, opalescent suspension which is described in this paper as a 1 : 25 sperm suspension. One drop of this mixture when added to one drop of eggs in 7.5 c.c. of sea water gives 100 per cent. membrane formation with no polyspermy and such an insemination is spoken of as a 1 : 25 : 7.5. insemination.

Concentrated stock solutions were made up as percentage of metallic salt in distilled water by means of the volumetric flask. The solutions used in the experiments were made up by the addition of the stock solution to sea water. Care was taken with those metals which required high concentrations, that the proportion of sea water and stock solution in the mixture used should not alter the osmotic pressure to any appreciable extent. Solutions were not used for more than five days save in the case of $AuCl_3$ and $PtCl_4$ where the rarity of the metal demanded the use of the one solution.

When an insemination is spoken of as immediate, *i.e.*, made in the solution itself, 7.5 c.c. of the solution was put into a watch crystal and one drop of eggs and one drop of 1 : 25 sperm suspension added at opposite sides of the dish. The dish was immediately rotated to insure thorough mixing of the gametes. In the viability tests, the eggs were normally inseminated in sea water and one drop of these was then added to 7.5 c.c. of the solution. The dish was then rotated as stated for the immediate inseminations.

A number of comparative tests were made in which, owing to the size of the experiment set up, it was necessary to use the eggs of a number of females. Unless so stated, each experiment was made with the eggs and the sperm of but one pair of individuals.

IV. Experiments.

I have chosen to deal with the experimental section of this paper in three parts. In the first I will take up in some detail the behavior of each solution which I used. I have taken the metals up in the order of decreasing toxicity. It will be noted that Mercuric chloride is apparently out of place, but as it gives no membrane inhibiting point in the same sense as the other metallic salts, it has been put at the end.

The series of tests described in part *b* was made for the purpose of comparison. The tests show the great similarity in the action of the various salt solutions on the eggs and the sperm, not only when they are unmixed, but also on insemination. The latter includes both immediate inseminations and viability tests.

Under *c* I have summarized the experimental data. There is also a table there that shows both the membrane-inhibiting concentration and the cleavage-toxic concentration for each of the metals.

a. Data on the Various Metallic Salts.

1. *Gold Chloride.*—On July 20, 1922, a 1 per cent. stock solution of $AuCl_3$ was made in distilled water and used for establishing concentrations in sea-water as shown in Table I*a*. These were prepared separately for, and at the time of each experiment. A series of tests was then set up to establish the effects of various concentrations on eggs which were inseminated immediately in the solutions. The results are shown in the accompanying Table I*a*. It can be seen on examination, that one part of $AuCl_3$ to 1,500,000 parts of sea-water will completely inhibit membrane elevation. At 1 : 600,000 and 1 : 300,000, narrow hyaline zones appear around the eggs after an exposure of about five minutes to the solution, but upon examining the control, it was found to be merely the first indicium of cytolysis in the egg.

A series of tests was then made with the purpose of determining what concentration is necessary to inhibit cleavage when the eggs are transferred to the solutions five minutes after insemina-

TABLE I*a*.

Concentration of $AuCl_3$ in Sea-water.	Per Cent. Membranes.		Per Cent. 1st Cleavage.	
	♀ *A.*	♀ *B.*	♀ *A.*	♀ *B.*
1 : 12,000,000	100	100	99	100
1 : 6,000,000	68	20	69	18
1 : 3,000,000	22	2	20	2
1 : 1,500,000	0	0	0	0
1 : 1,200,000	0	0	0	0
1 : 600,000	0	0	0	0
1 : 300,000	0	0	0	0
Uninseminated control in sea-water	0	0	0	0
Inseminated control in sea-water	100	100	100	100

Effect of $AuCl_3$ on eggs inseminated therein. July 20, 1922. Temp. 22.5° C. Eggs and sperm fresh. Insemination immediate 1 : 25 : 7.5.

tion in normal sea-water. The results for one of these tests is given in Table I*b*. Table I*b* shows the concentration of $AuCl_3$ toxic to cleavage to be 1 : 375,000. Comparison with Table I*a* brings out the difference between membrane-inhibiting and cleavage-toxic concentrations.

TABLE I*b*.

Concentration of $AuCl_3$ in Sea-water.	Per Cent. 1st Cleavage.	Per Cent. 2d Cleavage.
1 : 1,500,000	90	3
1 : 750,000	12	0
1 : 375,000	0	0
1 : 185,000	0	0
Inseminated control in sea-water	98	97

Test of cleavage-toxicity of $AuCl_3$. July 20, '22. Eggs from ♀ *A*. Opened 1 : 34 P.M. Membranes 100 per cent. Eggs transferred to solution five minutes after insemination in normal sea-water. 1 : 25 : 7.5. Sperm fresh. Temp. 24° C.

In Table I*c* can be seen viability tests run with the same lot of eggs at both the cleavage-toxic and the membrane-inhibitory concentrations. This test shows the difference between these two kinds of action. A concentration that is completely inhibitory to the initial events has no absolute effect on the subsequent events, while a more concentrated solution will prevent the occurrence of the subsequent events provided the time factor is sufficient. The effect of the exposure time is also shown by this table. The effect on cleavage is cumulative. The higher the concentration of the metallic salt, the shorter need the time of exposure be, to produce complete inhibition.

TABLE I*c*.

Time of Transfer after Insemination in Normal Sea-water.	Per Cent. Membranes.		Per Cent. 2 Cell.		Per Cent. 4 Cell.	
	A.	*B*.	*A*.	*B*.	*A*.	*B*.
Immediate	0	0	0	0	0	0
30 sec.	92	93	29	0	12	0
1 min.	95	95	46	0	14	0
2 "	98	98	80	2	65	0
4 "	100	100	84	4	68	0
8 "	100	100	84	16	80	0
15 "	100	100	92	24	86	0
30 "	100	100	96	78	95	0
Inseminated control in sea-water	100		98		99	

Viability tests. Aug. 12, '22. 5 : 27 P.M. Temp. 21° C. Eggs ♀ *B*. 5 : 10 P.M. Sperm fresh. Insemination 1 : 25 : 7.5. Solutions:

A, 1 : 1,500,000 $AuCl_3$.
B, 1 : 375,000 $AuCl_3$.

Lillie ('21) found that there are three ways in which egg when placed in the membrane-inhibiting concentration of $CuCl_2$ can be made to fertilize and produce membranes. Two of these are by means of protective agents. In the first case he used egg sea-water of high agglutinating power. In the second, he used a solution of gelatine or of gum arabic. The third method is by the use of an excess of sperm. A test was made of the effect of increased sperm concentrations on gold inhibition with the results shown in Table I*d*. A concentration of $AuCl_3$ of 1 : 750,000 was used in this experiment. Such a concentration has a p.H. of 8.0 and has no visible effect on the spermatozoa.

TABLE I*d*.

Drops 1 : 25 Sperm.	Per Cent. Membranes.	Per Cent. 1st Cleavage.
1	0	0
2	0	0
4	0	0
6	4	3
8	20	20
12	36	34
20	100	92 much polyspermy.

Effect of increased sperm concentration. Aug. 7, 1922. 10 : 38 A.M. Temp. 25° C. Eggs 9 : 50 A.M. Sperm fresh. $AuCl_3$ 1 : 750,000.

2. *Copper Chloride*.—Lillie ('21) investigated the effect of

copper salts of the fertilization reaction of *Arbacia* and found that there was a membrane-inhibiting point at 1 : 500,000 He also determined the cleavage-toxic concentration as 1 : 62,500. A repetition of this work confirmed the determinations that he made save that for one of the females employed it was found necessary to use a concentration of 1 : 37,500 $CuCl_2$ to completely inhibit cleavage. As has been stated above, the cleavage-toxic concentration varies somewhat, both for the eggs of different females and for the different physiological conditions existing in the eggs of the same female.

3. *Zinc Chloride.*—Zinc, it will be recalled, is one of the metals which formed such a precipitate that a cleavage-toxic concentration could not be determined. In the weaker solution necessary to completely inhibit the formation of membranes, however, the salt could be used with little difficulty. The usual series of experiments for the determination of this point was set up. The result of such an experiment may be found in Table II*a*.

TABLE II*a*.

Concentration of $ZnCl_2$ in Sea-water.	Per Cent. Membranes.	Per Cent. 2 Cell.	Per Cent. 4 Cell.
1 : 250,000	96	76	Present.
1 : 200,000	6	5	"
1 : 175,000	0	0	0
1 : 150,000	0	0	0
1 : 125,000	0	0	0
Inseminated control in sea-water	96	96	96
Uninseminated control in sea-water	0	0	0

Test of membrane-inhibition of $ZnCl_2$. July 5, '22. ♀ *C*. Eggs and sperm fresh. Insemination 1 : 25 : 7.5. Exp. 2 : 30 P.M. Insemination immediate. All membranes narrow.

Table II*b* is a record of the viability of eggs which were inseminated in normal sea-water and transferred to the $ZnCl_2$ solution at intervals as indicated. It will be noted that when good membranes are raised, the toxicity of the membrane-inhibiting concentration on cleavage is not very marked. When narrow membranes have been raised the viability is much poorer.

4. *Lanthanum Chloride.*—Gray ('15) found that cerous chloride in a very weak concentration, would aggregate the sperm of *Arbacia*. He also found that by the addition of a small amount

TABLE II*b*.

Time of Transfer after Insemination in Normal Sea-water.	Per Cent. Membranes.	Per Cent. 2 Cell.	Remarks.
Immediate	0	0	
30 sec.	95	63	33 per cent. narrow membranes.
1 min.	100	82	Memb. good.
2 "	100	98	" "
2 "	100	98	" "
4 "	100	100	" "
8 "	100	100	" "
15 "	100	100	" "
30 "	100	100	" "
Inseminated control in sea-water	100	100	

Viability test. 1 : 150,000 $ZnCl_2$. July 5, 1922. Eggs and sperm fresh. Insemination 1 : 25 : 7.5. ♀ *A*. Exp. 2 : 45 P.M. Temp. 20° C.

of NaOH, the sperm could be reactivated. Because of this, I made a few additional experiments on the sperm of *Arbacia* with $LaCl_3$ solutions. In the case of the immediate insemination in the lanthanum chloride solution, I found that, at a concentration of *N*/4,090 or 1 : 50,000, membranes are not raised. I then made up a thick sperm suspension by adding a number of drops of dry sperm to 7.5 c.c. of the $LaCl_3$ solution and observed that the sperm clumped and that their activity was much inhibited. The sperm in this solution were, however, reactivated by the addition of a drop of *N*/500 NaOH. I then made a similar suspension and added one drop of concentrated egg sea-water. The sperm immediately became very active and gave a very definite agglutination reaction.

After the membrane-inhibitory concentration had been established as 1 : 50,000, this solution was made up replacing the normal sea-water with concentrated egg sea-water, *i.e.*, egg sea-water of high agglutinating power. When eggs were inseminated in such a solution, the percentage of membrane formation rose from 0 per cent. to 64 per cent. to 72 per cent. It may thus be seen that egg sea-water has a great protective action in lanthanum-inhibition.

The viability test of the normally inseminated eggs in 1 : 50,000 $LaCl_3$ solution showed that this concentration is very slightly toxic to cleavage. As will be recalled, a cleavage-toxic concentration could not be determined because of the great amount of precipitation.

5. *Aluminium Chloride.*—The tests with $AlCl_3$ were much like those for the metals already described. The results were also much the same. It was found that a concentration of 1 : 40,000 (p.H. 7.2) would completely inhibit membrane elevation and that a concentration of 1 : 10,000 (p.H. of less than 6.6) was toxic to cleavage when the eggs were transferred to it after insemination in normal sea-water. As in the case of $LaCl_3$, the sperm were clumped when added to the solution, but when NaOH or egg sea-water was added, this clumping was corrected and the sperm became active. When the egg sea-water was added, the sperm agglutinated. Table IV. shows the result of a viability test on eggs inseminated in normal sea-water and later transferred to the membrane-inhibiting concentration of the salt. It will be noticed by an examination of this table, that the membrane-inhibiting concentration is more toxic to the first cleavage than is the case with the other metallic salts.

TABLE IV.

Time of Transfer after Insemination in Normal Sea-Water.	Per Cent. Membranes.	Per Cent. 2 Cell.
Immediate	0	0
30 sec.	80	20
1 min.	97	80
2 "	97	92
4 "	97	94
8 "	97	97
15 "	97	96
30 "	97	97
Inseminated control in sea-water	97	98
Uninseminated controls.		
a. Sea-water	0	0
b. $AlCl_3$	Eggs clumped.	

Viability test in 1 : 40,000 $AlCl_3$. June 19, 1922. ♀ *A.* Eggs and sperm fresh. Insemination 1 : 25 : 7.5. Exp. 1 : 00 P.M. Temp. 21.5 C.

A test of the increase of membrane elevation due to an increase of sperm concentration showed that the percentage was raised from 0 per cent. to 32 per cent. on increasing the amount of sperm from 1 drop to 15 drops of the 1 : 25 suspension in immediate inseminations.

6. *Platinic Chloride.*—In the case of no other metallic salt studied is there as sharp a line of demarkation between concentrations which permit of membrane elevation and those which are absolutely inhibitory, as with $PtCl_4$. A stock solution was

made from the crystals which was .9 per cent. in strength and all the concentrations were made up by adding this to sea-water. The test for the membrane-inhibitory concentration shows that at 1 : 16,666, there is membrane elevation to equal the control, while at 1 : 11,111, there is a complete inhibition of membrane elevation. The p.H. of the latter concentration is 7.4. In this case each egg may be surrounded by from twelve to sixteen very active sperm. They strike the membrane and remain agglutinated to it though they are unable to incite any cortical change whatever. The agglutination of the sperm, by a drop of egg sea-water, in such a $PtCl_4$ solution when the suspension is made up directly in the solution, is good. The inhibition comes after the agglutination and before the cortical response of the egg can show itself, *i.e.*, it occurs in the latent period.

Not only is there a sharp distinction between concentrations which allow complete membrane elevation and those which are absolutely inhibitory, in the case of this metal, but the membrane-inhibiting concentration and the cleavage-toxic concentration are both more rapidly toxic to the eggs than is the case with the other metals studied. Table V*a* shows the results of a viability test at the membrane-inhibiting concentration of this salt; Table V*b* shows the effect of such a solution on eggs which have been forced to produce membranes by the use of an excess of sperm; Table V*c* shows the result of a viability test at the cleavage-toxic concentration. All of these tests show the great poisonous effect of the solution on the egg. The test with the

TABLE V*a*.

Time of Transfer after Insemination in Normal Sea-water.	Per Cent. Membranes.	Per Cent. 1st Cleavage
Immediate	0	0
30 sec.	88	44
1 "	92	50
2 "	96	62
4 "	98	84
8 "	98	84
15 "	98	98
30 "	98	97
Inseminated control in sea-water	98	98
Uninseminated control in sea-water	0	0

Viability test in 1 : 11,111 $PtCl_4$. June 30, 1922. Eggs 9 : 00 A.M. ♀ *A*. Sperm fresh. Insemination 1 : 25 : 7.5. Experiment 10 : 00 A.M. Temp. 21° C.

TABLE V*b*.

Drops of 1 : 25 Sperm Suspension.	Per Cent. Membranes.	Per Cent. 1st Cleavage.
1	0	0
2	0	0
4	2 narrow.	0
8	70 "	0.5 irregular.
12	80 "	0
16	100 "	1
Inseminated control in sea-water	100 good.	100
Uninseminated control in sea-water	0	0

Effect of increased sperm concentration in immediate insemination in 1 : 11,111 $PtCl_4$. June 30, 1922. Eggs as in Table V*a*. Exp. 10 : 00 A.M. Temp. 21° C.

increased sperm concentration shows the resistance of this solution to enforced insemination as well. Only after the eggs have been left in normal sea-water for four minutes after insemination therein, does the viability in Table V*a* begin to approach the normal control. At this same concentration, 1 : 11,111, while 100 per cent. membrane elevation may be produced by increase of the sperm concentration from one to sixteen drops of the 1 : 25 suspension, only 1 per cent. of the eggs cleave and these are irregular.

TABLE V*c*.

Time of Transfer after Insemination in Normal Sea-water.	Per Cent. 2 Cell.	Per Cent. 4 Cell.
10 min.	0	0
20 "	0	0
30 "	0	0
45 "	2	0
Inseminated control in sea-water	96	96

Viability test in 1 : 1,945 $PtCl_4$. July 1, 1922. Eggs 2 : 00 P.M. Sperm fresh. Insemination 1 : 25 : 7.5. Membranes 100 per cent. Experiment 3 : 30 P.M. Temp. 22° C.

A test of the cleavage-toxic point gave that determination as 1 : 1945. As soon as the eggs were transferred to such a solution after insemination in normal sea-water, they become crenate. Such a solution allowed no cleavage up to 45 minutes when the eggs were transferred to it at intervals after insemination in normal sea-water. The results of this experiment are given in Table V*c*. Cleavage took place in 48 minutes in the controls.

7. *Lead Chloride.*—$PbCl_2$, when added to sea-water precipitates very freely. This precipitate is not so heavy, however, that the membrane-inhibiting concentration cannot be determined as 1 : 4,000 or *N*/554 (p.H. 7.0.). Some precipitate was present

even at this concentration so that the amount of the salt really in solution could not be figured. Sperm will agglutinate readily in such a solution, though the eggs become clumped.

8. *Nickel Chloride.*—A series of tests, made with $NiCl_2$, follow so closely the general behavior of the metallic salts already described that but little discussion is necessary here. It was found the membrane inhibition first becomes evident at about 1 : 30,000 when the percentage of membranes raised fell from 100 per cent. to 80 per cent. in immediate inseminations, while at 1 : 5,000, $N/324$, membrane elevation is completely supressed. Such a solution, when used in a viability test, shows a decided effect on the production of cleavages other than the first. The effect is shown not only by the percentage of cleavage, but also in its regularity.

When tests were made to establish cleavage-toxicity, the concentration required was found to be 1 : 600 or $N/39$ —. Agglutination of the sperm is good in 1 : 5,000 $NiCl_2$ when one drop of the egg sea-water is added to 7.5 c.c. of thick sperm suspension made up in the membrane-inhibiting concentration. The sperm are active in such a solution even before the addition of the egg sea-water.

9. *Cadmium Chloride.*—Cadmium differs from the other metals studied in only one respect and there the difference is quantitative and not qualitative. When eggs are placed in the membrane-inhibiting concentration, which is 1 : 1,333 + or $N/122.1$, and after various exposure times, are transferred to normal sea-water and inseminated, there is a very marked poison effect after two minutes exposure. Not only does two minutes exposure cause very irregular development, even at the first cleavage, but it tends to inhibit the elevation of membranes. After four minutes, there were 98 per cent. membranes formed and 12 per cent. were narrow. After one hour, all were narrow. When eggs were exposed to the solution for one hour and then insemiated, the first cleavage gave 16 per cent. regular forms and 42 per cent. irregular.

The effect of this concentration on the sperm is very marked. They agglutinate readily with egg sea-water and can be activated by one drop of $N/500$ NaOH or egg sea-water though they are quiescent in the solution alone. The percentage of membranes was raised from 0 per cent. to 84 per cent. by increasing the sperm concentration at insemination from one drop of 1 : 25 to 12 drops of the same suspension. The higher sperm concentration of this test resulted in much polyspermy.

It would be expected that by increasing the concentration of the $CdCl_2$ in solution, a cleavage-toxic point could be determined. I was successful in this; such a concentration is 1 : 250, or N 23 −.

10. *Cobalt Chloride.*—When a series of tests was made to establish the concentration of $CoCl_2$ necessary to inhibit the elevation of membranes, and the concentration necessary to inhibit completely the formation of the first cleavage in eggs which have been inseminated in normal sea-water and transferred to the solution five minutes after the insemination, it was found that both are much higher that in the other metals with which I worked. The membrane-inhibitory point was found to be 1 : 1373, or $N/89.245$, while cleavage-toxicity occurred at 1 : 100 or $N/6.5$. As was the case with Cadmium, though not to such an extent, the toxicity of even the membrane-inhibiting concentration on eggs which had been inseminated in normal sea-water and transferred to it, was very marked.

Agglutination is good in the solution. The membrane-inhibiting concentration makes the sperm sluggish but this harmful effect is corrected by the addition, of the NaOH or a drop of the concentrated egg sea-water.

11. *Mercuric Chloride.*—As has already been stated above, there is one metallic salt which is irregular in its behavior. This is Mercuric Chloride. All the other salts with which it has been possible to work have a double effect on the eggs of *Arbacia.* The first effect is at the weaker concentration and is on the phenomenon of membrane elevation; the second effect is constantly at a greater concentration and is dependent on the time of exposure of the gametes for the intensity of its action. It modifies materially the viability of the eggs and I have termed it "cleavage-toxicity." The tests made with $HgCl_2$ show that there was no concentration which would inhibit the elevation of membranes and allow cleavage if eggs were placed in it five minutes after they were inseminated in normal sea-water.

TABLE VI*a*.

Concentration of $HgCl_2$ in Sea-water.	Per Cent. Membranes.	Per Cent. 1st Cleavage.
1 : 1,000,000	100	16
1 : 800,000	100	12
1 : 600,000	100	0
1 : 400,000	100	0
1 : 300,000	100	0
1 : 200,000	100	0
1 : 100,000	52	0
1 : 75,000	48	0
Inseminated control in sea-water	100	100
Uninseminated control in sea-water	0	0

Test with $HgCl_2$. July 11, 1922. 11 : 20 A.M. Eggs and sperm fresh. Insemination 1 : 25 : 7.5. Eggs ♀ *C.* Temp. 20° C. Concentrations greater than 1 : 300,000 showed cytolysis in 20 minutes.

For example, an examination of Table VI*a* will show that at 1 : 600,000, $HgCl_2$ is toxic to cleavage, while there is 100 per cent. elevation of membranes which are wide and to all appearances absolutely normal. In those concentrations in which cleavage does not follow membrane elevation, cytolysis commences within an hour for those eggs with membranes. Eggs without membranes are more resistant. It appears from Table VI*b* that at 1 : 7,500 $HgCl_2$, membranes are not raised. At first glance it would be thought that this is a direct inversion of the ratio found to hold for the other metals where membrane-inhibition occurs at a lower concentration than cleavage-toxicity. An examination of the effect of such a solution on the sperm shows that they are instantly paralyzed by it and that this paralysis is not reversible, *i.e.*, neither NaOH nor concentrated egg sea-water will activate the sperm in it. This immediately differentiates such membrane-inhibition from that described for the other metals.

Lillie ('21) notes that the action of $HgCl_2$ is very different from that of $CuCl_2$. He notes that "the initial stages are relatively little affected, . . . the susceptibility increases as fertilization progresses" (page 140). He also notes that "mercury also suppresses the movements of the spermatozoa at great dilution." According to his data (Table VII.), a concentration of 1 : 625,000 completely inhibits cleavage while 1 : 15,625 prevents membrane elevation. He states further, that at the latter concentration, sperm are "paralyzed instantly." My observations are in accord with the above, save that it was found necessary to use a concentration of 1 : 7,500 $HgCl_2$ to instantly paralyze sperm and consequently to completely inhibit membrane elevation (Table VI*b*). Lillie considered that the action of mercury on membrane-inhibition was like that of copper but at a much higher concentration. I would prefer to consider that the action of $HgCl_2$ is a poisoning of the sperm, and not the type of inhibition that is present in copper-inhibition.

TABLE VI*b*.

Concentration of $HgCl_2$ in Sea-water.	Per Cent. Membranes.	Per Cent. 1st Cleavage.
1 : 75,000	86	0
1 : 50,000	70	0
1 : 25,000	40	0
1 : 20,000	30	0
1 : 15,000	20	0
1 : 10,000	4	0
1 : 7,500	0	0
Inseminated control in sea-water	98	99
Uninseminated control in sea-water	0	0

Test of $HgCl_2$. July 21, 1922. 3 : 04 P.M. Temp. 26° C. Eggs ♀ *C.* Eggs and sperm fresh. Insem. 1 : 25 : 7.5.

In most of the metallic salt solutions, the effect is to make the membranes narrow as the concentrations approach the strength necessary for membrane-inhibition; $HgCl_2$ has exactly the opposite effect. When eggs are inseminated in solutions which do not paralyze the sperm, membranes elevate immediately and such membranes are extremely wide. The solutions alone will produce membranes on eggs in concentrations greater than 1 : 150,000 as has also been observed by Lillie ('21). These membranes form much later than those produced by insemination so that it is easy to differentiate between the two. Eggs with membranes cytolyze very rapidly when left in $HgCl_2$ solution.

It would thus appear that, in comparing the action of the Mercuric salt with those of the metals already described, the only great difference is in the failure of the $HgCl_2$ to show any concentration which prevents the action between viable egg and sperm and consequently the initial response of the cortex of the egg.

b. Comparative Tests.

The preceding part is devoted to individual tests on the behavior of various salts. These tests were made with separate lots of eggs, under different conditions, and are therefore not as suitable for comparison as they would be had they all been done simultaneously with one set of eggs. Three sets of experiments were therefore set up for the purpose of comparison.

The experiment which appears in Table VII*a*, was made to show the effect of the various solutions on the viability of the same lot of eggs under the same conditions. In each case the concentration used is that of membrane-inhibition. In this table it will be noted that only the figures for the first cleavage are given. In all save the immediate inseminations, membranes were present on a high percentage of the eggs. The table, therefore, emphasizes the great difference between the inhibition of initial and subsequent events, and shows the great similarity of the action of all of the salts. In this table, the figures for $AuCl_3$ and $CuCl_2$ are interpolated. It was found, however, that both of these membrane-inhibiting concentrations proved more toxic to cleavage than those of the other salts. I have not used $HgCl_2$ in this test as it shows no membrane-inhibiting point.

Table VII*a*.

Time of Transfer after Insemination.	Percentage First Cleavage. Chlorides of:									
	Au[1]	Cu[1]	Zn	La	Al	Pt	Pb	Ni	Cd	Co
Immediate	0	0	0	0	0	0	0	0	0	0
30 sec.	29	38	87	98	94	96	80	70	80	80
1 min.	46	64	96	98	92	95	84	78	87	92
2 “	80	89	98	100	97	96	92	90	98	94
4 “	84	98	100	100	100	98	98	92	100	98
8 “	84	99	100	100	100	99	100	98	100	99
15 “	92	99	100	100	100	100	100	100	100	99
30 “	96	99	100	100	100	100	100	100	100	99
Inseminated control in sea-water	98	99	100							

Comparative test with membrane inhibitory concentrations. July 15, 1922 Experiment 10 : 15 A.M. Temperature 21° C. Sperm and eggs fresh. Insemination 1 : 25 : 7.5. Membranes formed well, save in immediate inseminations.

[1] In the above experiment, the results for Au and Cu are interpolated.

The experiment summarized in Table VII*b* was performed to determine the time when superficial cytolysis first begins to appear in uninseminated eggs placed in membrane-inhibiting concentrations of each of the metallic salts. Readings were made at the end of fifteen minutes, one half hour, and at half-hour intervals thereafter for four and one-half hours. In this experiment, one drop of the uninseminated eggs was placed in 7.5 c.c. of the membrane-inhibiting concentration of each of the metallic salts. The container, which was a Syracuse watch-glass was then rotated to insure a thorough mixing of the gametes and the solution.

Table VIIb.

Metallic Salt.	Superficial Cytolysis Begins. Hours.
$AuCl_3$	$\frac{1}{4}$
$HgCl_2$	$\frac{1}{2}$
$CuCl_2$	1
$ZnCl_2$	$1\frac{1}{2}$
$LaCl_3$	2
$AlCl_3$	2
$PtCl_4$	$3\frac{1}{2}$
$PbCl_2$	$3\frac{1}{2}$
$NiCl_2$	$3\frac{1}{2}$
$CdCl_2$	$3\frac{1}{2}$
$CoCl_2$	4
Control in sea-water	None in $4\frac{1}{2}$ hours.

Test of time of cytolysis in membrane-inhibitory concentrations. Eggs, 1 drop to 7.5 c.c. solution. 10 : 45 A.M. 7/26/22. Eggs fresh, 2 ♀s. Temp. 22.5° C.

Table VIIc.

Metallic Salt.	Action Alone.	1 Drop *N*/500 NaOH Added.	1 Drop Concentrated Egg Sea-water Added.
$AuCl_3$	Active.	Very active.	Very active. Agglut.
$CuCl_2$	"		" " "
$ZnCl_2$	"	Paralyzed.	" " "
$LaCl_3$	Inactive. Clumped.	Clumped. Some activity.	" " "
$AlCl_3$	Active. Clumped.	Clumped. Very active.	" " "
$PtCl_4$	Active.	Very active.	" " "
$PbCl_2$	Active. Some clumping.	" "	" " "
$NiCl_2$	Active.	" "	" " "
$CdCl_2$	Quiescent.	" "	" " "
$CoCl_2$	Sluggish.	Fairly active.	" " "
$HgCl_2$	Paralyzed.	No change.	No change.

Effect of membrane-inhibitory concentrations on the sperm. July 18, '22. Sperm suspension made with dry sperm in 7.5 c.c. of the solution. Sperm from two males.

The experiment in VII*c* was with the membrane-inhibiting concentration of each of the metals and its effect on the sperm.

In this series, a sperm suspension was made by adding a number of drops of the dry sperm to 7.5 c.c. of the membrane-inhibitory concentration of the metallic salt, and the effect of the solution on the behavior of the sperm was noted. A drop of NaOH (*N*/500) was then added to one preparation and its effect noted. To another preparation, a drop of concentrated egg sea-water was added. The results may be seen in Tab'e VII*c*.

c. Summary.

In summarizing the results of the foregoing experiments, it may be generally stated, that, with the exception of $HgCl_2$, all of the metals studied have three effects on the fertilization period of *Arbacia*. The first and second occur at a greater dilution than the third and involve membrane elevation. When eggs are inseminated directly in certain solutions, they give no cortical response, *i.e.*, the effect is immediate and prevents any reaction between the gametes themselves; if, however, eggs are placed in this same solution after cortical discharge has begun but before it is complete, a "narrow membrane" results which may be attributed to an incomplete cortical response. The third involves the subsequent events and requires a higher concentration of the metallic salt. It seems from the data to be cumulative in its action. When the eggs are placed in the solutions five minutes after the insemination in normal sea-water, the time factor amounts to approximately forty minutes, as first cleavage is used as an indicator. If the eggs are transferred at a later time, first cleavage may be only partially inhibited but there will be no second cleavage. That is to say, the time required for the salt solution to produce its toxic effect is, in these experiments, about thirty-five or forty minutes. Table VIII gives a comparison of the concentrations necessary to produce these effects. The order of toxicity of the metallic salts will be seen to be the same for both membrane effects as well as subsequent events. The general results are in accord with those noted by Lillie in his work on copper. Mercury, as has been observed above, is an exception to this general rule.

In each case, as can be seen by reëxamination of Table VII*c*, sperm may be caused to agglutinate in the membrane-inhibitory

TABLE VIII.

Metallic Salt.	Membrane-inhibition.		Cleavage-toxicity.	
	Parts.	Normality.	Parts.	Normality.
$AuCl_3$.....	1 : 1,500,000	*N*/151,800	1 : 375,000	*N*/37,912.5
$CuCl_2$.....	1 : 500,000	*N*/ 42,030	1 : 62,500	*N*/ 8,405
$ZnCl_2$.....	1 : 150,000	*N*/ 10,215		
$LaCl_3$.....	1 : 50,000	*N*/ 4,090		
$AlCl_3$.....	1 : 40,000	*N*/ 1,780	1 : 10,000	*N*/ 445
$PtCl_4$.....	1 : 11,111	*N*/ 936	1 : 1,945	*N*/ 163.87 —
$PbCl_2$.....	1 : 4,000	*N*/ 554		
$NiCl_2$.....	1 : 5,000	*N*/ 324	1 : 600	*N*/ 39 —
$CdCl_2$.....	1 : 1,333.33	*N*/ 122.1	1 : 250	*N*/ 23 —
$CoCl_2$.....	1 : 1,373	*N*/ 89.245	1 : 100	*N*/ 6.5
$HgCl_2$.....			1 : 600,000	*N*/81,000

The above table shows the series of metals as established for both membrane and cleavage inhibition in *Arbacia*. With the exception of $HgCl_2$ they are in the order of increasing concentration.

concentrations by the addition of egg sea-water, thus demonstrating that the action is not primarily on the sperm before their interaction with the female gametes.

V. DISCUSSION.

There are three periods definable in *Arbacia* from the moment of insemination up to the first cleavage. The first involves the behavior of the spermatozoön alone and includes its activation, migration to the egg, and its agglutination to the egg. The second involves the reaction of both egg and sperm, and lasts from the end of the latent period to the completion of the cortical activation of the egg. It can, of course, be subdivided. The third is concerned with the rotation of the sperm head within the egg cytoplasm, and the migration of the egg nucleus. In other words, it is the preparation for the union of the germ nuclei and the preparation for cleavage. A latent period comes between the time of the agglutination of the sperm to the egg and the initial events of cortical activation, as described by Lillie ('21). It belongs to the first period described rather than to the second.

The differentiation between the first and second periods is very markedly emphasized by the foregoing experiments. When eggs are placed in solutions indicated in the text as membrane-inhibitory, and immediately inseminated, the sperm are seen to agglutinate to the egg. This inhibition, therefore, very evidently occurs between the periods of agglutination and of activation, or during the latent period. It may be said that the egg is held at the latent period by the solution. As soon as the agglutination is seen to occur, there is a block interposed, and the reaction ceases. At the time when this block is first interposed, there is no great harm done to the eggs. If they are removed to sea-water and inseminated, the sperm penetrate and cortical activation ensues. This seems to show that the action involved affects only the very external parts of the gametes and is easily removed. After an exposure of slightly longer duration, the viability of the eggs becomes poorer, but this I would consider to be due to the penetration of the salt into both the cortex, and, later, the central part of the egg and consequently, to a very different mechanism from that which prevents the elevation of the membrane.

There are a number of physical and chemical phenomena which may be responsible for this inhibition of membrane formation. The theory of chemical combination was suggested by Lillie. He remarks ('21) the similarity of the effective concentrations found to hold in the "cleavage toxicity" of his work and in the enzyme poisoning discussed below. The physical action may be of two kinds. It may be an adsorption phenomenon resulting in either a change of the electrical charge on the membrane which is present before fertilization or an adsorption by molecular groups. Heesch ('21) finds that the charge on cell membranes of *Lycopodium* spores, leucocytes, and yeast cells can be reversed by the use of $La(NO_3)_3$ solutions. Whether this action is the same as that in membrane-inhibition or not, is an open question. Membrane-inhibition might be due to a change of charge of the egg membrane, or to an adsorption of the metallic ions by some of the complex molecular groups in the egg protoplasm. Were these groups those of the activable substance, this adsorption would explain the partial activation described above. The first of these would involve an electrical action while the latter would result in a chemical inactivation though not a chemical reaction.

Lillie ('21) noted the close similarity between the concentrations of $CuCl_2$ required for cleavage-toxicity and that found by v. Euler and Swanberg ('20) to be toxic to the action of saccharase. McGuigan ('04), in an extensive set of tests of the effect of the metallic salt solutions on the action of diastase, determined the concentrations which inhibit its action for a series of metals. Olsson ('21) also worked on the effects of the various salts of silver and copper on the action of amylase. v. Euler and Swanberg ('21—II) found that the action on saccharase by organic substances was not effected by the temperature. They conclude that there is a binding of the enzyme through the aldehyde group. McGuigan compares the action of the salts to the solution tensions of their ions, though he finds certain irregularities in the series. His results for diastase and those of v. Euler and Swanberg for saccharase are in very close agreement.

The data concerning membrane-inhibition in *Arbacia* do not agree with the results noted by the above authors for enzyme-poisoning. While chemical activity could explain the inhibition effect satisfactorily, it would be difficult to conceive an immediate correction of this, effect if the inhibiting factor were removed. As has already been noted, when the eggs are removed to sea-water from a solution in which inhibition is complete, if the exposure has not been too long, there is an immediate recovery and the eggs will produce membranes on insemination in the normal manner.

The present paper emphasizes the distinction to be made between the initial events in fertilization as they are indicated by the cortical activation, and the subsequent events which lead to the period of cleavage.

VI. Conclusions.

1. The chlorides of the following heavy metals inhibit membrane formation in *Arbacia* in the following order and concentrations;—Au—*N*/151,800, Cu—*N*/42,030, Zn—*N*/10,215, La—*N*/4,090, Al—*N*/1,780, Pt—*N*/936, Pb—*N*/554, Ni—*N*/324, Cd—*N*/122.1, Co—*N*/89.2. The effect of these concentrations upon the cortical response of the egg is immediate. Eggs inseminated 1 : 25 : 7.5 in these solutions immediately become bombarded by many sperm, no one of which is able to activate the cortex of the egg at the above concentration.

2. In contrast to the above, but showing the same general order, are the concentrations of the same metallic salts which are toxic to cleavage. These salts, with their toxic concentrations are;—Au—*N*/75,825, Cu—*N*/8,405, Al—*N*/445, Pt—*N*/163.87, Ni—*N*/39 –, Cd—*N*/23 –, Co—*N*/6.5. There was so much precipitation in the case of Zn, La, and Pb, that the concentration of these salts, toxic to cleavage could not be determined.

Mercuric chloride proved toxic to cleavage at *N*/81,000, while seeming to favor membrane elevation. At a concentration inhibiting membrane elevation, sperm were paralyzed immediately.

These concentrations vary slightly for different batches of eggs and show the influence of a time factor, thus giving an interesting contrast to membrane-inhibition, the concentrations of which are more constant and immediate in their action. These concentrations are greater in every case except Hg, than the membrane-inhibiting concentration of the same metal. Cleavage toxicity is a progressive, or a cumulative action.

3. These solutions are not immediately harmful to the gametes, but prolonged exposure is injurious to the eggs as can be shown by two sets of experiments.

(*a*) When the female gametes are exposed to the solutions for various periods of time and are then inseminated in fresh sea-water, the viability always stands in inverse ratio to the length of exposure.

(*b*) When the inseminated eggs are transferred to the solutions at intervals after insemination in normal sea-water, the length of exposure to the solution bears an inverse ratio to the viability of the eggs.

It will be seen in both (*a*) and (*b*) that the time factor is the important one. The action here is again cumulative.

4. With the exception of mercuric chloride, any harmful effect of these solutions on the sperm is corrected by the addition of a drop of egg sea-water.

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THE BEHAVIOR OF CALCIUM PHOSPHATE AND CALCIUM CARBONATE (BONE SALTS) PRECIPITATED IN VARIOUS MEDIA, WITH APPLICATIONS TO BONE FORMATION.

JAMES CRAWFORD WATT,

ANATOMICAL LABORATORY, UNIVERSITY OF TORONTO.

WITH THIRTY-EIGHT FIGURES IN THREE PLATES.

CONTENTS.

PAGE
Introduction 280
Part 1. Microscopic Study of Precipitation:
  Technique 282
  Reaction in aqueous solutions 283
    Precipitation of calcium phosphate 283
    Precipitation of calcium carbonate 284
  Precipitation in colloidal solutions 284
    Precipitation of calcium phosphate 285
    Precipitation of calcium carbonate 285
  Influence of other agents on the precipitate 288
  Influence of different ions 290
  Precipitation in blood serum 291
  Precipitation in hyaline cartilage extract 291
  Influence of hydrogen ion concentration 292
  Disintegration of spherules 295
  Number of forms assumed by precipitate 297
Part 2. Examination of Bone:
  Technique and material 298
  Results of examination of bone 300
Part 3. Discussion:
  Theories regarding deposit of calcium salts 301
  Reversibility of calcium reaction 305
  Summary 307

INTRODUCTION.

There are at present three main theories presented in explanation of the manner in which the matrix of bone becomes impregnated with the two inorganic salts, calcium phosphate and calcium carbonate. These views may be briefly summarized as follows:

1. That the salts are deposited in the matrix by a precipitation *in situ* from the interaction of soluble salts in the blood and the tissue.

2. That the salts are excreted, or secreted, either with the matrix, or into the matrix, by the bone cells.

3. That a complex combination salt known as calcium carbonophosphate, carried in solution in the blood, is thrown out of solution in the bony matrix by a change in the carbon dioxide content of the tissue, and after precipitation is finally converted into the two components, calcium carbonate and neutral calcium phosphate, in the exact proportions found in bone.

The weight of evidence is at present in favor of the third view, especially supported by the work of Barillé and of Wells, but it seems to me that fully conclusive proof that this is the process has not yet been brought forward.

The precipitation theory, however, offers a very simple and plausible explanation, and so it was determined to investigate the behavior of bone salts on precipitation and see if any light could be shed on the actual manner of impregnation of the bony matrix.

An exact knowledge of the way in which the bone salts are added would be a great aid, indeed, to our understanding of the growth of bone, repair of fractures, changes in rickets and osteomalacia, and other kindred conditions. It would also throw light on the formation of calcareous patches in scar tissue and in arteriosclerosis, for Wells has shown that calcification and ossification are quite similar processes, and the same two calcium salts are present in both cases in exactly the same proportions.

This research work, as finally carried out, involved the microscopic study of the reactions whereby calcium carbonate and calcium phosphate were precipitated, both in separate solutions and also in the same solution, first in various aqueous media and then in certain colloidal ones. This was followed by a thorough examination of the unchanged matrix of many different bones in the mouse, the frog, the guinea pig, the dog, and human fœtuses of various ages.

"For experimental investigation of the artificial process will furnish the best clue to a precise and certain knowledge of the natural one, by showing more clearly how much is due to physical agency"—Rainey.

PART I. MICROSCOPIC STUDY OF PRECIPITATION.

TECHNIQUE.

For simple examination of a reaction, a glass slide measuring 38 x 75 mm. was taken and on it were outlined two squares with sides of 20 mm. painted in melted paraffin, so that the wax formed the sides of a shallow cell. Into a cell were placed a small amount of the two reacting solutions to give the desired precipitate, and then a cover glass 22 mm. square was dropped on, supported by the wax cell wall. If it was desired to keep the specimen, melted wax was then painted all around the edges, and overlapping on to the cover glass. The wax was then coated with thick shellac, which in its turn overlapped slightly on to the glass. The cell was thus permanently sealed. Two cells could be made on one slide and the same reaction could be compared in distilled water and in a colloidal solution side by side.

It was found desirable to examine not only the precipitate formed immediately at the line of contact of the two reacting solutions, but also what was formed later, in more remote parts of the solution, during diffusion, and as the concentration of the dissolved salts became less and less with increasing precipitation. To do this required larger cells, and it was desirable to have a fairly uniform thickness of cell, which was accomplished as follows:

On a glass slide 38 x 75 mm. in size two thin parallel strips of mica dipped in melted paraffin were so placed as to support the long edges of a glass cover slip measuring 24 x 50 mm., which was placed upon them. These long edges were now thoroughly painted over with melted paraffin, which on hardening cemented the cover slip to the slide. A cell measuring from 0.1 mm. to 0.2 mm. deep was thus made, still open at both ends. By means of a fine glass pipette one of the reacting solutions, $M/10$ calcium chloride, was now introduced at one end until the cell was nearly two thirds filled. This end was then carefully dried and sealed with paraffin.

At the still open end was now introduced the other solution—for instance, $M/4$ sodium carbonate, or $M/4$ triple sodium phosphate—and the last border of the cell was then sealed and the slide labeled. All borders were then painted with thick shellac, which was allowed to slightly overlap the wax on to the glass.

To obtain a series of cells of uniform depth, a sheet of mica four inches by two inches in size was split until the required thickness was obtained, equal to that of a number two cover glass. It was cut across with scissors into two-inch strips about two millimeters wide. One sheet thus prepared will cut into approximately forty or fifty strips.

Cells filled with warm solutions sometimes showed broken cover glasses on cooling. Also in cells completely filled with solutions a slight amount of expansion due to change of temperature was sufficient to cause leakage by loosening the wax from the slide. And so to provide for permanency of the specimens all cells were so filled as to leave an air space to provide for contraction or expansion without undue strain, and were filled with solutions at room temperature, 20°–21° C.

Pure distilled water was used in all experiments, all solutions were filtered to make them perfectly clear, and chemically pure reagents were employed in every case.

Reaction in Aqueous Solution.

Precipitation of Calcium Phosphate.

Calcium phosphate was obtained by the interaction of $M/10$ solution of calcium chloride and $M/4$ solution of triple sodium phosphate in water. This strength of reagent gave plenty of precipitate, without it being so dense as to interfere with proper microscopic examination. As the two solutions were of different concentration, there was a different rate of diffusion on the two sides of the line of contact, with corresponding differences in rate and amount of precipitation.

Immediately that contact occurs between the two solutions a milky cloud appears, which grows rapidly in size. For a moment, although the cloud is visible to the naked eye, the solution seen microscopically is still clear, but an instant later it becomes violently agitated, and the agitation becomes quickly defined as being caused by very minute particles, which are in very rapid motion, both oscillatory and translatory. These particles, appearing only as small dots even under the highest magnification, grow in size, losing their motion as they increase in bulk, until finally they form the fine amorphous granules which gather in clumps, and masses, and fine films, to form the precipitate.

Precipitation of Calcium Carbonate.

Calcium carbonate was precipitated by the interaction of a $M/10$ solution of calcium chloride with a $M/4$ solution of sodium carbonate. The process here resembles that described above for calcium phosphate in the form of myriads of fine particles in rapid Brownian movement. All movement finally ceases as these particles agglutinate into large clumps, and then a remarkable change occurs, for the granular masses just formed fade from the sight, dissolving again into the solution, while scattered here and there larger, rapidly growing particles occur, which become defined as crystals, angular in outline, or as small spherical bodies, known as spherules, which are later converted into crystals as they increase in size (Figs. 1, 2, 3, and 25).

Two or three hours after the formation the precipitate is all crystalline. It is very rare to see a spherule persisting in pure aqueous solutions.

The appearance of precipitates of calcium carbonate in the form of spherules has been frequently noted before. As long ago as 1839 it was described by Link, who, in accordance with this phenomenon, thought that crystals at their first origin were fluid and only later became hard and angular, as solidification occurred.

PRECIPITATION IN COLLOIDAL SOLUTIONS.

The colloids employed were gelatin and egg albumen.

Various concentrations of gelatin were experimented with, ranging from an 8 per cent. solution, which solidified quickly, to a 1 per cent. solution, which remained fluid for some time and then formed a soft jelly. As the various concentrations gave similar results the weaker ones were used as a matter of routine, being easier to handle.

For albuminous solutions Merck's powdered soluble egg albumen was employed, water being added to make the approximate normal proportions given by Lillie for fresh albumen in the hen's egg, namely, albumen 12 per cent., water 88 per cent. by weight.

In 1857 Rainey published the first of a series of papers on the precipitation of carbonate and phosphate of calcium in colloids, using gelatin, albumen, and gum arabic. His best results were obtained with gum arabic, and he pointed out that the solutions of the gum holding the two salts reacting to give the precipitate should be of very different densities, so that diffusion and the consequent reaction would occur slowly and gradually. I have found, however, with albumen and gelatin, that if the reacting salts differ sufficiently in concentration, the concentration of the colloidal solution need not differ in order to secure good reactions. And so in many series of experiments 1 per cent. gelatin was used throughout, although in others this concentration was used only with calcium chloride, and a 4 per cent. solution with sodium carbonate or phosphate. Egg albumen was used always in the same concentration.

Precipitation of Calcium Phosphate in Colloids.

The employment of a colloidal solution seems to have little or no influence on the precipitation of calcium phosphate. The deposit is quite similar to that in aqueous solutions, being fine, granular, and amorphous (Fig. 36), and aggregating to form thin gelatinous veils and clouds. Results in egg albumen were similar to those in gelatin. These findings are in agreement with the work of Rainey, Harting, Biedermann, and others.

Precipitation of Calcium Carbonate in Colloids.

The reaction here is very characteristic. Calcium carbonate formed in the presence of colloids has a tendency to separate out and persist in the form of the spherules mentioned previously, which have been named calcospherites.

The first effect of the colloid is to very much emphasize and prolong the stage in which the particles are small and show active Brownian movement, apparently by preventing their early fusion into larger masses. In a short time, however, larger particles are seen which grow partly at the expense of the smaller ones, which redissolve as described for aqueous solutions. But there are many small particles which apparently do not dissolve, but pass on directly over to the larger form of deposit, by forming a nucleus for the laying down of additional material from the clear solution.

The precipitate exhibits two separate forms. One of these is distinctly crystalline (Figs. 8, 10, 12, 13) and is found most densely at the primary line of contact of the reacting solutions where deposit was very rapid. Even here the colloid shows its influence, for the crystals are not perfect, but are deformed in various ways, and exhibit rounded angles and suppression of their typical shape.

The second form of deposit is in the shape of very perfect small spheres (Figs. 7–12, 29, 30, 31), which vary greatly in their transparency, markings, and size according to the solution in which they are formed.

Rainey obtained spherules in gum arabic only after the lapse of an hour or more. At first a faint nebulosity was seen, lasting in thin solutions of gum for one hour, in thick solutions for over a week, after which spherules occurred. In my work I have been able to identify these bodies in less than ten minutes, and in some solutions to see them attain a size of 30 μ in one hour. They grow rapidly and attain practically their full size in forty-eight hours, ranging then according to the solution in which they lie, from a size of 1 μ up to 120 μ. In Rainey's case they grew gradually for several months and ranged in size from 2 μ to 200 μ. I had one single example of a pear-shaped body which measured 120 μ x 300 μ.

Evidently gum arabic exhibits a more powerful colloidal influence on the precipitate, but gelatin or albumen will give excellent results in a very much shorter time, which is a valuable consideration.

Brownian movement in parts of some slides is still active after the lapse of a year, due to the colloid retaining some of the precipitate in its original, extremely small, granular form, preventing coalescence to form the larger particles seen in other areas where precipitation was denser and more rapid.

The process of coalescence of the small particles can be observed in favorable cases. Very small spherules showing active movement travel about in the solution, colliding with and working past others. Suddenly two of these, instead of passing, will be drawn together, as though pulled by a strong attractive force, and will merge completely in one large spherule, which in its turn may absorb small ones. At these moments of coalescence the spherules give the observer the impression of being fluid, flowing together like two drops of water, and lead one to believe that Link was correct in interpreting these small spherules as liquid crystals. As these bodies become larger they lose their movement, settle down, and become of glassy hardness. There is also a later growth by addition of material from the surrounding clear solution.

Richards and Archibald have shown that the particles of precipitates at their first appearance seem to have a rounded outline to the eye even under high magnification, but by photographs, however, they have demonstrated that these particles are real crystals of typical shape. This condition is true for some particles of calcium carbonate formed in aqueous solutions, but in colloids a great deal of the deposit is in the form of particles which are round, and persist in this shape as they grow. There are other particles also, which clearly are not round, and give the crystalline form of deposit which occurs simultaneously with the spherules.

The spherules formed in gelatin (Figs. 13 and 31) are very perfect, with a smooth surface, and clear, transparent, glassy appearance. Those formed in albumen (Figs. 26 and 29) often show a slightly irregular periphery and give indications of their internal structure. They appear to be built up of concentric layers, and often exhibit an amorphous center. Extending from this center to the periphery is also a faint, but quite distinct, radial striation in certain cases. These appearances have already been well described and illustrated by Rainey, Harting, and Biedermann.

It ought to be noted here that Harting has been erroneously credited by many writers with the discovery, in 1872, of the calcospherites formed in colloidal solutions. Rainey described and gave accurate illustrations of these bodies in 1857, and had observed them as early as 1849, and so deserves the credit of this discovery.

Combined precipitates of calcium carbonate and phosphate (Fig. 36) show each salt acting quite independently, the phosphate occurring in its usual amorphous form, the carbonate in typical crystals and spherules. Similar conditions were found by Rainey in gum arabic.

The colloids gelatin and albumen were chosen as being artificial media easily prepared which might be looked on as approximately comparable respectively to the bony matrix and to the blood serum or to cellular tissues. Reactions in gelatin might give a clue to those in bone, and similarly reactions in albumen might indicate processes of calcification in other tissues. After studying the reactions in these simple media, they were further modified by the addition of various substances which are either normally or pathologically found in the blood or tissues, to see what effect these special substances would exert.

Further experiments were also carried out to study the effect of the hydrogen-ion concentration of the colloid. To complete this stage of the work reactions were followed in cartilage extract and in blood serum. Then specimens of bone from various animals were examined to see if there was any correlation between the structure of their matrix and the results obtained experimentally in the various media.

The Influence of Other Agents on the Precipitate.

The substance whose influence was to be investigated was added in equal concentration both to the sodium carbonate or phosphate, and to the calcium chloride solutions which were to react to form the precipitate. The new agent was thus balanced so that its action could not be one sided.

The agents used and their concentration in solution were:

$M/10$ sodium chloride, same proportion as in human blood serum.
0.1 per cent. dextrose, same proportion as in human blood serum (Gradwohl).
0.05 per cent. urea, same proportion as in human blood serum.
0.1 per cent. lecithin. Blood serum contains 0.7 per cent. (Wells).
1.0 per cent. acetone. Simulating conditions in acidosis.
0.5 per cent. ethyl alcohol.

The effects produced on the precipitation of calcium carbonate were quite definite for some of these substances, and in some instances very remarkable changes of form resulted. The influence on calcium phosphate was much less striking, as this deposit remained in the same granular, amorphous form in all cases, and the results were mainly a slight difference in the average size of granules, and also differences in the speed and completeness of precipitation similar to those which will be noted in detail below for calcium carbonate, to which latter deposit alone the succeeding paragraphs relate.

Addition of Sodium Chloride.

The main effect noted on adding sodium chloride was the speeding up of the reaction. Brownian movement ceased sooner, crystallization was more rapid, and in most cases the main portion of the precipitate consisted of smaller crystals and spherules, and in the colloids the crystals were of very poor shape.

Addition of Dextrose.

The influence of this compound was slight and seemed to consist mostly in added transparency of the crystals and spherules. There was also slight modification in shape.

Addition of Urea.

Even in aqueous solutions some spherules were formed when urea was present. The effect in colloidal solution (Figs. 30 and 35) was to favor increase in size of the spherules, with a marked tendency toward fusion, so that many double forms and dumbbell-shaped bodies were seen. Urea thus shows an influence quite the opposite of sodium chloride upon the precipitate. It evidently favors growth of this deposit and suggests the idea that the presence of urea in blood and in urine may aid in the deposit of calcium salts in the calcareous incrustation of sclerotic arteries and the growth of renal and vesical calculi.

Addition of Lecithin.

Lecithin, even in small amounts, 0.1 gm. in 100 c.c. water gives a turbid solution even after filtering, but microscopically the solution appears clear, so that it is evidently a colloidal one.

The lecithin has a remarkable influence on the precipitation of calcium carbonate. Even in aqueous solution (Figs. 4, 5, 6) spherules occur in great numbers, and many of these are of a large size not obtainable in other solutions. There is also a distinct tendency toward fusion of spherules in contact with each other, so that double and multiple forms are numerous. Beautiful rosettes (Fig. 5) were present, due to the adhesion of many oval-shaped bodies.

In gelatin and albumen (Figs. 10 and 11) the same phenomena occurred, producing magnificent rosettes and very large spherules measuring 120 μ in diameter.

Lecithin thus has a very typical colloidal action on the precipitate, favoring the spherical forms of deposit. This compound is found in blood in seven times the strength it was employed here, and is also found in bone and other tissues, so that its action on a precipitation of bone salts, if such occurs, ought to be appreciable. This point will be discussed later.

Addition of Acetone.

The presence of acetone (Fig. 12) hastens the process of precipitation, making it occur very rapidly, and also makes the initial deposit at the moment of contact much denser and more complete. This point is rather suggestive. It leads to the question as to whether or not conditions of acidosis such as accompany serious cases of nephritis and diabetes have an influence favoring the progress of calcification in the arteriosclerosis so often found in these cases.

Addition of Ethyl Alcohol.

The alcohol had no appreciable effect upon precipitation except to add to the optical clearness of some preparations.

The Influence of Different Ions.

The calcium carbonate was precipitated in separate cells by the carbonates of sodium (Figs. 1 and 4), potassium (Figs. 2 and 5), and ammonium (Figs. 3 and 6), so that the solutions contained the three last-named ions as the only variable, all other factors being equal. In aqueous solution the crystal form of the calcium carbonate in the three cases was noticeably different. In colloidal solutions the spherules seen were very different in their appearances in each set of slides.

The most noteworthy change was seen where the ammonium salt was used, the deposit being much coarser, with rougher, more irregular surfaces, and as a rule much greater in size and with more tendency toward fusion and toward clumping into masses.

The whole series for each ion, including those slides with the six accessory agents described above, showed similar tendencies to the plain, unmodified reaction. Each series consisted of 21 slides, consisting of 7 in water, 7 in gelatin, and 7 in albumen.

Precipitation in Blood Serum.

Clear serum was pipetted off from test tubes of dog's blood allowed to stand for some hours in a refrigerator. The precipitation of calcium phosphate in this serum showed the usual granular amorphous character. Calcium carbonate separated out almost entirely in the form of spherules, many of which were quite large. The process of precipitation in blood serum was very complete, covered practically the whole area of the slide, and seemed to be greater than usual in amount. This appearance suggested the thought that the sodium carbonate and phosphate of the blood aided the reaction, making precipitation greater in amount, but further consideration made this seem unlikely, as the usual amount of carbonate or phosphate was added to the blood, and this alone was sufficient to precipitate more than the total amount of calcium, without the aid of the salts naturally in the blood.

Mixtures of carbonate and phosphate present the usual character, each salt being precipitated in its own peculiar form and being thus easily identified (Fig. 36).

Precipitation in Hyaline Cartilage Extract.

An extract of fresh hyaline articular cartilage was prepared by mashing it up in distilled water, allowing the mixture to stand a short time, and then filtering it and using the clear solution. The same concentrations of salts were used here as in other cases, but the resulting precipitate was very much less in amount. Precipitation occurred only in the area of contact of the solutions, was in a finely divided state, showed some tendency to form crystals, but mostly was in form of granules and very small spherules. There were no large spherules whatever. The total amount of deposit would lead one to think that very weak solutions had been employed instead of the usual ones.

Hyaline cartilage extract thus seems to decrease the amount of precipitation and to retain what deposit is formed in a very finely divided state. Addition of the various accessory substances used in previous cases had no effect, even lecithin apparently being powerless to produce the large spherules.

These facts may have some significance, for here we have an extract of a tissue which although apparently similar to other cartilage which does ossify, and although situated in continuity with ossified tissue, yet retains a matrix free from a deposit of calcium salts. Its extract forms the most unfavorable medium yet found in which to produce precipitation of calcium salts. Solutions of equal concentration in other media give three to four times the amount of precipitate found here. The experimental facts, it seems, are in complete accord with the nature of the tissue, which is unfavorable to the deposition of calcium salts in it.

Influence of Hydrogen-Ion Concentration.

Loeb's method was employed of treating gelatin to obtain it with a definite hydrogen-ion concentration. To 0.25 gm. gelatin in a test tube were added 20 c.c. of cold acid solution of a definite strength, and this was allowed to stand for 30 minutes, during which time the gelatin swelled remarkably, but did not dissolve. It was then filtered and washed on the filter paper four times with 25 c.c. of cold distilled water at 5° C. to remove all free acid.

The gelatin was then transferred to a test tube standing in hot water, where it melted, and the volume was made up to 25 c.c. with hot distilled water, thus giving a one per cent. gelatin solution. Two tubes for each strength of acid were prepared. On cooling the pH of each pair was determined approximately by Clark's colorimetric method, and then one tube of the pair was rendered a $M/10$ calcium chloride solution, and the other one a $M/4$ sodium carbonate solution, and these two solutions were placed as usual in a cell to react.

There was also an alkaline series made similarly to the above. In each series there were ten grades as follows:

Acid series treated with $\frac{1}{16}, \frac{1}{32}, \frac{1}{64} \cdots \frac{1}{8192}$ HCl.

Alkaline series treated with $\frac{M}{16}, \frac{M}{32}, \frac{M}{64} \cdots \frac{M}{8192}$ NaOH.

The pH of the acid series of washed gelatins in one per cent. solution ranged from 3.2 to 6.0. Untreated gelatin, both in sheets and in powder, which had been used for all the previous experiments was found to possess a pH of 6.0.

The weakest alkaline member of the series registered 6.2 and the fifth member of the series a pH of 9.0, above which no record was kept.

The amount of precipitate visible to the naked eye was less in the acid series than the alkaline one. It was least of all in those specimens where the pH was in the neighborhood of the isoelectric point of gelatin, which occurs in the acid series at 4.7, and which marks the point where the gelatin is most insoluble and most inert. In these slides there was practically no diffusion and spreading of the precipitate, only a narrow band of deposit occurring along the line of contact of the reacting solutions. Such a condition would lead one to infer that the salts in solution are intimately bound to the gelatin.

It was also noted that crystals (Figs. 17, 18, 19) were abundant, and very often large, in the various acid gelatins. Crystals were also present in the weaker alkaline specimens, but in gelatin treated with $M/2048$ NaOH or stronger alkaline solutions (Fig. 23) not a single crystal has yet appeared in a period of six months since the preparation of these slides. Here the precipitate is still in the form of very small spherules, often so densely packed as to prevent Brownian movement, but elsewhere still actively in motion, which has gone on continuously for six months without a change, and gives every indication of continuing indefinitely. The larger of these spherules, approaching one micron in diameter, have a slow movement and so can be well observed, and have given definite proof of what was already suspected from a study of other slides, namely, that the so-called spherules are in reality flattened discs. During their vibratory movement many of them have been observed to slowly roll over and a narrow, thin edge is presented to the view instead of the circular outline. The appearance reminds one very strongly of a red blood corpuscle.

The largest and clearest spherules (Figs. 19–22) were formed in gelatin whose pH was between 4.7 and 7.5—that is, all in gelatin on the alkaline side of the isoelectric point—but in specimens whose reaction was either weakly acid or weakly alkaline, the neutral point being 7.0. This is the zone in which is obtained the formation of the largest spherules. Untreated gelatin, used for all the previous series of reactions without regard to hydrogen-ion concentration, has a pH 6.0 and so is within this zone. The pH of human blood and many tissues also lies in the alkaline part of this zone.

It is interesting to note that in strong acid members of the series precipitation is rapid, and while spherules may early be present, the deposit in the course of a short time becomes entirely crystalline in structure. In strongly alkaline members precipitation is very complete, but the deposit remains for an indefinitely long time in the form of small, discrete spherules, which do not grow or fuse, and which continue to exhibit Brownian movement. No crystals appear in these latter cases, even after the lapse of many months.

In the intermediate zone of weak acid, neutral, or weak alkaline gelatin both spherules and crystals appear in quantity, and the spherules are very large. In the part of this zone in which the hydrogen-ion concentration of blood and many tissues lies the deposit is mostly in the form of small spherules and the tendency to crystallization is very poor. This is essentially a colloidal phenomenon and seems significant in view of the fact that it has frequently been found that certain drugs in colloidal form have a much better therapeutic effect, have a much less damaging effect on healthy tissues, and are much more pleasant in their administration to the patient. As an example of this, compare the actions of silver nitrate and of argyrol, a colloidal silver vitellin. The colloidal preparation much more nearly approaches the condition of the tissues themselves and the reactions ought to be of a more colloidal character, which I have shown is the natural condition with the hydrogen-ion concentration on the basic side of the neutral point. To the acid side of this point reactions tend to the ordinary chemical crystalline variety.

DISINTEGRATION OF SPHERULES.

A most remarkable phenomenon was observed during the last two months in some of the slides, where spherules have been observed in process of disintegration and transformation into crystals. This was first noticed in the acid gelatin series in a slide where the gelatin had a pH of 4.9 and where for the whole of six months appearance had been constant.

This case (Fig. 31) had offered one of the most perfect examples of large spherules, which were of perfect outline, glassy transparency, absolutely clear, and showing no indication at all of their internal structure. These spherules were formed in half an hour and had attained their full growth in less than two days, and for six months presented the same appearance, with no indications of a change. It happened at the end of this period that they were not examined for about one month, when it was discovered that some of them (Figs. 19 and 33) had disappeared, others were disintegrating, and all still present were opaque, while in this area which had not previously shown a single crystal there was now a number of large crystals.

Other slides were examined for similar conditions without avail, but a month later several of them showed signs of a change, and by examination of these at intervals a complete series of stages in the transformation has been obtained and a complete description of it can now be given.

The first sign of a change is in the occurrence of a very fine opaque central spot of a bluish tint (Figs. 20, 21, 24*a*, and 32) in the otherwise clear spherule. This area increases a little in extent, and the next stage then is ushered in by the appearance around this center (Fig. 24*b*) of a faint nebulous haze, which becomes more opaque and finally seems to consist of a fine radial striation (Fig. 24*c*) which extends farther and farther, until it reaches the periphery of the spherule, which is now an entirely opaque white body. At this stage some spherules are uniformly opaque (Fig. 24*d*), others show a marked radial striation (Fig. 24*e*).

The final step in the change now occurs with a very gradual disappearance of the spherule, apparently by dissolving into the surrounding solution. It decreases in diameter and also in thickness until the remnant, diminished very much in size, appears like a nebulous ghost of the former spherule and eventually fades completely from view. During the solution the opaque center of many spherules seems to dissolve early (Figs. 24*f* and 32), converting the body into a ring.

In those cases where the spherule is uniformly opaque (Figs. 24*d* and *f*) solution is very uniform at the surface, the contour of the spherule remaining perfect while the size contracts. Where there is radial striation, however (Fig. 24*e* and *g*), the periphery dissolves unevenly, giving the spherule a rough, rather ragged surface.

Coincident with the beginning of the process of dissolving of the spherules, crystals occur in these areas which formerly were entirely composed of spherules, and which for several months' duration had not shown a single crystal. These crystals grow steadily both in size and in number (Figs. 19, 20, 21), while the spherules disappear. The passage of the material from the spherule into solution is evidently very transitory, it coming out again immediately to be deposited in the crystal.

What is the interpretation of this phenomenon? It seems to me that there is a very evident one, and that this is the same process very much delayed in starting, and very much protracted in its action, which occurs rapidly in ordinary aqueous solutions. It will be remembered that in precipitating calcium carbonate in pure water it is at first in a granular, amorphous form, which later redissolves and comes out of solution a second time as spherules and crystals, which grow rapidly, the spherules all being converted into crystals during growth.

Again in colloidal solutions the same process happens, but is delayed somewhat, taking hours or days instead of minutes. In such cases an area of spherules seen one evening after a precipitation, when examined next morning is found to consist of crystals.

Apparently in some of these cases at least, as Brownian movement ceases and the particles grow in size, the spherule is transformed directly into the crystal.

In the degeneration of the large spherule it seems to me we have a similar phenomenon to that of the other cases. The large spherules could correspond to the amorphous particles of the aqueous solution, fused into a large mass, the redissolving occurring very late to form the final crystal. There is evidently a very strong directive force toward the formation of crystals even in the colloids, and though interfered with and hindered by them for a long period, it nevertheless conquers. What it is that initiates the change after the deposit has remained stationary for many months is problematical. There was no infection in the gelatin, no change in its appearance, or any other fact to give a clue.

Some slides in other series in both gelatin and albumen also show evidence of some change in the spherules, which in view of the above facts may be expected to progress farther.

As far as I know this remarkable transformation of the precipitate has never been described before. The further investigation of this phenomenon is outside of my province and belongs to the field of physics and chemistry, where it will provide some very interesting problems which I hope some competent investigator will undertake to solve.

A second method of disintegration (Fig. 6) has also been observed in a lecithin solution, where some large spherules with very marked striation lost this striation after the lapse of several months and became dully opaque. Certain clear radii then appeared along which cleavage occurred, splitting the spherule into sectors which finally separated, giving triangular crystals.

Number of Forms Assumed by Precipitate.

It is to be remarked that in no slide was it possible, after Brownian movement ceased, to obtain only a single form of deposit. There were always two, and in many cases several forms occurring, and this applies equally to pure aqueous and to colloidal solutions. This multiplicity of form in the precipitate has also been noted by Biedermann and is due doubtless to differences in the relative concentration of the reacting salts and in the diffusion currents in different parts of the solution. Side by side may be found an area of crystals and of perfect spherules, or both forms may be seen mixed in one area.

In passing from the sodium carbonate end of a cell across the precipitate to the calcium chloride end there is often seen a marked change in shape of the deposit, forms present to one side of the line of contact of the two solutions not being present on the other. Spherules may occur all to the one side, but follow no rule as to which they select. Transition forms occur from one area to another. Orde found similar conditions on sectioning plugs of coagulated albumen with which he had sealed the ends of capillary tubes containing solutions which gave precipitates in the plugs when the sealed ends of the tubes were immersed in certain other solutions.

The multitude of forms exhibited in the precipitate of calcium carbonate in the slides seemed remarkable, but on referring to Goldschmidt's "Atlas der Krystallformen," I found illustrations for 2,544 modifications of the crystal form of calcite which have already been observed. Many of the changes are slight, but all are quite evidently different, and among them may be recognized all the types of crystal form I have obtained. Spherules and their variations were not shown.

PART 2. EXAMINATION OF BONE.

Technique and Material.

The ordinary method of examining bone by means of very thin ground sections, or by sections cut from decalcified bone, are useless to give the structure of the matrix. A method just published by Bast, however, for the study of bone cells *in situ,* without cutting sections, is equally applicable for the examination of the matrix and was here employed.

Bast worked with the parietal and ethmoid bones of mice and young rats, rabbits, cats and dogs, the nasal conchæ of these animals, pieces of the ethmoid in man, and other bones thin enough to be transparent. These were mounted whole. The specimens were fixed in 95 per cent. alcohol, washed in water, stained for

8 hours or more in diluted aqueous solution of Gentian violet, dehydrated in alcohol, cleared in benzol, and mounted in balsam.

To stain the bone cells only, the periosteum was left on the specimens, and this was later removed by dissection under a binocular microscope while the bones were in benzol. In this way the matrix remained clear, while if the periosteum was removed previous to staining, the matrix stained also.

This method was used by me, but as the matrix was the main object of study, most of my preparations were not stained, but were mounted for observation clear, in three different ways: first, without fixation, in a shallow cell filled with normal saline; second, in a shallow cell of glycerin; and third, fixed in 95 per cent. alcohol, then absolute alcohol, cleared in benzol, and mounted in balsam.

A cover glass to which some of the precipitate of calcium carbonate was adherent in the form of both spherules and crystals was passed through all the processes necessary to staining above and mounted in balsam. There was no change to be seen in the precipitate, so it can be inferred that calcium salts in the bony matrix, if in this form, would not be altered by any of the processes.

The specimens of bone used were parietal and ethmoidal bones and nasal conchæ of the mouse, frontoparietal bones of the frog, and splinters and thin sections in various planes from the femur, tibia, humerus, radius, ulna and scapula, of frog, mouse, guinea pig, dog, and human fœtus. The frog and mouse were young, so that their bones were in process of growth, and if there is any visible difference between new formed and old matrix it should be evident here. Thin pieces of the long bones cut with a sharp knife to take in areas of the total thickness of the shaft, or to show transition from the bony shaft to the cartilaginous epiphysis, were taken, splinters were clipped off long bones, small pieces were crushed, and all these were mounted as described above. The whole of a concha, scapula or parietal, if small, was mounted as one specimen.

In all cases the periosteum was carefully peeled off and was mounted alongside of the bone so that examination of it for newly formed matrix could also be made.

Results of Examination of Bone.

Both bright and dark field illumination were employed and specimens of bone, when thin, were quite transparent and easily examined. In bone that is well formed, whether from adult, or young individual, or fœtus, the appearances are similar. The bone cells and all their processes in the canaliculi are easily followed and appear as Bast has described them. The matrix round about them is homogeneous, transparent, and almost glassy in appearance, and gives no indication (Fig. 37) of being formed of separate particles. If it was originally formed in such a manner, there has been complete fusion, so that there is no evidence of a precipitation. In fact, the whole appearance bears out the statements of text-books of histology that, although two thirds of the bony matrix is composed of inorganic salts, they are so intimately blended with the organic material that there is no visible evidence of their presence.

In the case of growing long bones, such as those of the limbs, sections were taken across the shaft to include the newly formed growing periphery just under the periosteum, and also sections in the length of the bone to include the end of the diaphysis, the epiphyseal plate of cartilage, and the bony epiphysis. These sections, however, were disappointing, as even at the transitional areas any tissue that could be identified as bony matrix appeared homogeneous, so that no evidence of precipitation could be obtained here.

In the case of the developing diaphysis of the long bones of the limbs in human fœtuses, however, there were definite, discrete particles to be seen. In Quain's Anatomy there is a fine description by Schäfer of the appearances of developing bone with which my findings entirely agree. Just ahead of the advancing bone of the diaphysis is an area of change, where the tissue is no longer cartilage, but is not yet true bone. Here we find definite fibres (Fig. 38) like those of white fibrous tissue, and known as Sharpey's fibres, extending from the bony material forward into the cartilage, and in these fibres in the narrow area immediately in advance of the true bone are numerous very fine granules which appear similar to the material of the matrix in well-formed bone and so are evidently the bone salts. They are crowded in the fibres just ahead of the actual bone, and in the bone itself we find the homogeneous appearance due to the fusion of these discrete particles into one mass. Pacchioni has also described the first appearance of lime salts in bone in the form of fine granules which later form a homogeneous mass.

The possible origin of these particles either by precipitation or secretion will be discussed later.

PART 3. DISCUSSION OF RESULTS.

Concerning some of the physical phenomena of the precipitation reactions studied, remarks have been made at appropriate places in the previous pages describing the work and need not be repeated here.

There are a few points to be constantly borne in mind in considering the question of ossification, which have been very clearly demonstrated by Wells. The most important is the fact that there is a very definite and fixed ratio between the amount of calcium carbonate and calcium phosphate deposited in bone. The second point is that this same ratio is equally true in areas of calcification in tissues other than bone. The third fact is that the processes of ossification and of calcification are essentially the same, the kind of matrix or tissue in which the deposit occurs differentiating the two processes.

Theories Regarding Deposition of Calcium Salts.

The reactions with calcium phosphate show it to be invariably precipitated in a granular form so that if it were visible in bone it should be evident in a finely divided amorphous form. As this salt forms over eighty per cent. of the inorganic material, it ought to be readily demonstrated, but this is not the case, because the matrix is clear and homogeneous, not granular, except at the advancing edge of ossification in developing fœtal bones.

It has been shown that in mixed solutions calcium carbonate is not prevented, by the presence of the phosphate, from coming out in typical spherules or crystals, so that it might be expected that the carbonate would show in one of these forms in bone, in which it forms fifteen per cent. of the salts. This expectation is further strengthened by the fact that typical spherules have been found in the shells of invertebrates, which are mostly calcium carbonate, by

Rainey and by Biedermann. The dentine and enamel of the teeth, Rainey believed he had demonstrated to be composed of spherules, forming in rows and coalescing. Neither in old bone nor in new, nor in a transitional area transforming from cartilage to bone, have I seen either a single crystal or a single spherule.

Previous work has demonstrated that spherules can always be found in calcium carbonate precipitated in colloids. This formation is favored by the occurrence of the reaction gradually and slowly, and also by the presence of lecithin, which is found in both blood and bone, so that all the conditions in the matrix of the bone are favorable to the formation of spherules if precipitation occurs. The hydrogen-ion concentration is also favorable to formation of large spherules. With optimum conditions for their occurrence, and with a complete absence of these bodies, the interpretation is that the salts found in the bony matrix are not deposited in it by simple precipitation from a double decomposition of salts in the blood or the tissue. This is purely negative evidence, which, of course, is not of the same value as positive evidence, but it seems to me forms a very strong point against the simple precipitation theory.

It must be noted, however, that if calcium carbonate is very small in amount compared to the amount of phosphate precipitated that spherules may not be in evidence, but the whole precipitate will be granular, with coarser granules, resembling minute spherules and interpreted as carbonate interspersed among the masses of finer granules formed by the phosphate. It must be emphasized that homogeneous masses such as seen in the bony matrix never occurred in any of my experiments, but precipitates, no matter how long they were kept, remained composed of distinct discrete particles.

Another point against this theory is that it supposes that a soluble salt of calcium circulates in the blood to be precipitated as phosphate and carbonate in the bone. With the large amount of sodium carbonate and sodium phosphate in the blood, calcium carbonate and phosphate should be formed at once, here and not in the bone. But calcium is present in the blood in a greater amount than calcium carbonate and calcium phosphate are soluble in water.

And so it might appear that calcium can not be carried in the blood in the form in which it is later deposited in the bone. Pauli and Samec, however, have shown that difficultly soluble salts like calcium carbonate may have the amount of their solubility in water increased to over seven-fold in an albuminous solution, and so it is possible that the bone salts, formed by interaction of the blood salts and calcium absorbed into the blood as calcium chloride, might still be carried in solution in the blood and deposited in the bone.

This is closely related to the views of Barillé, whose theory is a step in advance of this, and is strongly supported by such work as that done by Wells. According to this view the calcium is in the blood in the form of a double salt formed here, tribasic calcium carbonophosphate, $P_2O_8Ca_2H_2:2CO_2(CO_3H)_2Ca$. This is soluble in the blood concentration of carbon dioxide. Infiltrating tissues like the bony matrix where the carbon dioxide content is lowered, this is precipitated and immediately breaks up into calcium carbonate and dicalcium phosphate, which latter salt is converted into calcium phosphate, giving the final proportion found in bone of 15 parts calcium carbonate to 85 parts phosphate. Even by this method of precipitation, however, one might expect to be able to see the salts in the matrix, and not being able to do so argues in favor of their being combined with the matrix. The most important point brought out in this theory is a clear consistent explanation supported by experimental evidence, as to the fixed proportion in the amount of the two calcium salts, the carbonate and phosphate, which is always found in their deposition.

It is interesting to note here the following facts regarding cartilage. Wells has shown that there is an almost specific power in cartilage for the absorption of calcium salts. My work is in agreement with this, for in cartilage extracts there was obtained the least amount of precipitation obtained in any of the experiments, thus showing the power of the substances in cartilage to hold on to the calcium. In spite of this affinity for calcium, however, microscopic evidence of developing ossification in bones preformed in cartilage shows us that the deposition of calcium is never directly in a cartilaginous matrix, but that the cartilage preceding the bone is destroyed and replaced by the organic bony matrix which is of a fibrous character, and this then has the calcium salts deposited in it.

The final possible explanation of the deposit of bone salts in the matrix is the secretory one. Wells and others have been forced to the conclusion that there is a selective or specific action of tissues, such as cartilage or membrane about to ossify, by which calcium salts are absorbed or otherwise taken into them, while apparently entirely similar areas of cartilage and membrane do not ossify at that time or at any later time. From this view of selective action it is not a far step to that of secretory action, explaining the ossifying process as a secretion of the calcium salts by the bone forming cells. It is already generally admitted that the matrix is a product of the bone cells, and if this is true, it is quite logical to assume that the calcium salts are taken by the bone cells from the blood and passed on into the matrix.

The difficulty in this theory has been to account for the amount of phosphate in the tissues. The calcium was looked upon as being derived from that carried in the blood, but the phosphate was explained as being due to cellular activities, derived either from the nucleus or from cellular degeneration. Another difficulty here, again, is to explain the fixed ratio of carbonate and phosphate. This, of course, could be looked upon as due to the carbon dioxide content of the tissue, or as due to the activity of the cell, a definite proportion being found in the secretory products here just as in other organs of the body.

It seems to me, however, that the nearest approach to the truth is to be obtained by combining two views. The bone salts, I believe, reach the tissue as the soluble double salt, tribasic calcium carbonophosphate, which by its constitution provides for the tissue both calcium carbonate and calcium phosphate, each salt in the proper proportion found in bone. I do not believe, however, that deposit here is by precipitation, for in the bone, owing to the colloidal matrix, the hydrogen-ion concentration, and the presence of lecithin, all conditions are most favorable to a visible and characteristic precipitate, showing granules and spherules, but there is no visible evidence whatever of these bodies. The only evidence in favor of precipitation consists of the following two sets of facts:

First, Schäfer and Pacchioni have described the first appearance of bone salts in developing bone in the form of minute granules which quickly coalesce. This I have confirmed. This evidence could also be interpreted as showing the presence of the salts to be due to secretory activity. Secondly, Olivier in a tumor of the breast and Pettit in a tumor of the maxilla and also in a renal cyst saw and made illustrations of calcospherites. These bodies can be explained only as a precipitation phenomena, but they do not afford sufficient evidence to prove that this is the process whereby the bone salts are deposited. And so these salts, after reaching the bone in correct proportions as the double salt above mentioned, must be deposited in the matrix by some more subtle process than ordinary precipitation.

Here we have recourse to the secretion theory. The bone salts in their proper proportions having been brought to the cell by the blood, are taken by the cell and secreted as an integral part of the matrix in combination with the ossein, the organic base, which it is generally admitted already, is a product of the cells. This will produce a matrix uniform in appearance, with salts invisible, and provides the proper proportion of each salt, as the cell is in its turn provided with the two salts in that exact amount. Histological evidence shows that the fibrous matrix appears first in the newly forming bone and impregnation with the bone salts begins immediately, the salts showing as fine granules at first, and coalescing as their quantity increases, to form a homogeneous matrix.

Reversibility of Calcium Reaction.

There seems to be evidence that this process of secretion is reversible, the bone cells being able to take up the calcium salts again. Wells and others have shown that calcium is removed from the bones in cases where there is a great demand for it in the body, as in pregnancy, or where it is being steadily lost by the body, for instance by way of a pancreatic fistula. Also, the osteoclasts responsible for the normal resorption of bone are supposed by some authors to be only osteoblasts which have changed their function. And further, where a graft of either living or dead bone, or a bone plate and bone screws are used to repair an injury, a process known as creeping replacement occurs whereby this material is gradually absorbed and replaced by new bone. It has been shown by Gallie and Robertson that it is the osteoblasts which invade the graft, gradually absorb the old matrix and bone salts immediately surrounding them, and then replace this by newly formed substance.

If the bone salts were laid down by precipitation of tribasic calcium carbonophosphate due to a change of the carbon dioxide content, it seems to me it would be impossible for this precipitate to be removed again as easily as Wells states it is, in response to the body's need for calcium. Would pregnancy or a pancreatic fistula so change the carbon dioxide content of the bone, as to make possible a reversal of the conditions that brought about the precipitation, and so either provide for increased solubility of the two salts or else make possible the recombination of the carbonate and phosphate of calcium into the double salt, with its consequent solution and carrying off in the blood?

It seems to me that the carbon dioxide content in the bone will not vary enough to permit of such a reversal, as to raise it would indicate a high activity in a tissue whose metabolism is low. This low-grade activity is given as a reason for obtaining the precipitate.

The taking of the calcium salts out of the bone probably depends on some balance that has to be maintained between the blood, the osteoblast, and the bony matrix. If the blood is rich in calcium, the cell can take it and pass it on into the matrix. If the blood is poor in calcium, the cell to maintain its relation in balance to the blood takes from the matrix to add to the blood. This is an instance comparable to the relations between the blood and its dextrose, and the liver cell and its stored glycogen, to maintain a certain carbohydrate balance.

This view also follows logically upon that of Wells, who regards the bony matrix as a great reserve store of alkaline bases for the body, where the calcium salts are in a state of flux, being constantly added to or drawn upon according to the needs of the body. Changes in the bones are much greater in extent and more rapid in their occurrence than is generally believed. It seems to me that this idea of the reversibility of the direction in which calcium salts pass through the tissues is a most valuable one for the proper comprehension of some of the processes of growth, repair of fractures, and changes going on in rarefaction of bony tissues.

SUMMARY.

1. Calcium phosphate precipitated in water or in colloidal solutions is constantly granular and amorphous in character, and apparently uninfluenced by the nature of the solution.

2. Calcium carbonate precipitated in water shows a great diversity of crystalline form; in colloidal solutions it shows two main forms, an irregular crystalline one and a spherical.

3. Mixtures by simultaneous precipitation of both salts in the same solution show each salt separating out independently and the part of the deposit formed by each can be easily identified.

4. Spherule formation by calcium carbonate is a typical reaction in colloidal solutions.

5. Spherules and crystals are influenced in shape, size, number, and internal structure by a variety of substances found normally or pathologically in the blood, most notably by lecithin which favors the formation of large spherules, and by acetone which increases the rapidity and extent of precipitation.

6. The character of the deposit of calcium carbonate is influenced by the hydrogen-ion concentration of the colloidal solution, being most crystalline in acid media, all in the form of spherules in strongly alkaline media, and mixed in form in solutions neutral or nearly neutral.

7. Large spherules, after persisting for months may undergo a sort of degenerative process, with change of internal structure, after which they dissolve and the material forming them is laid down in a crystalline form. As far as I know this phenomenon has never been previously described.

8. In fresh bone of various animals examined in various ways there is no visible microscopic evidence of the bone salts, although they form two thirds of the mass of the matrix. The inference is that the bone salts are not deposited in the matrix by simple precipitation, for the conditions are such that, if precipitated, granules, spherules, and crystals should be visible.

9. In the rapidly developing fœtal skeleton the first appearance of bone in the matrix is in the form of fine granules or globules which quickly fuse or coalesce to form a homogeneous mass. This might be interpreted according to the bias of the observer as supporting evidence either of precipitation or of secretion of the salts into the matrix.

10. The view advanced by Barillé and supported by Wells's work, that calcium is carried in the blood as tribasic calcium carbonophosphate, is probably correct, as it furnishes the bone salts in the proper proportion, but their view of its deposit in the matrix as a precipitate due to change in concentration of carbon dioxide does not appear correct in view of the fact that no precipitate of the bone salts is visible.

11. The theory that the salts furnished by the blood are taken by the bone cells and secreted by them along with the matrix seems reasonable in view of the condition found in the matrix.

12. The action of the osteoblasts seems to be reversible, they being able to absorb or take up the calcium salts again out of the matrix.

In concluding this paper I wish to acknowledge my indebtedness to others by thanking Professor J. Playfair McMurrich for special apparatus and for valuable references, also the following for providing material for investigation: Professor W. H. Piersol, Dr. R. D. Defries, Dr. Banting, Dr. Simpson, Dr. Dafoe, and Dr. Low.

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EXPLANATION OF PLATES.

PLATE 1.

All figures are from camera lucida tracings and are of equal magnification, × 200, so that the relative size of precipitated particles in each case can be compared. Only forms which were common, and appeared in quantity have been shown.

All illustrations are to show forms assumed by calcium carbonate precipitated from various solutions by the reagents listed below.

FIG. 1. Crystals of calcium carbonate precipitated by sodium carbonate in distilled water.

FIG. 2. Crystals of calcium carbonate precipitated by ammonium carbonate in distilled water.

FIG. 3. Crystals of calcium carbonate precipitated by potassium carbonate in distilled water.

FIG. 4. Deposit of calcium carbonate precipitated by sodium carbonate in distilled water containing 0.1 per cent. lecithin. Note small spherules, and small crystals, a tendency towards clumping, a very large spherule and a large rosette.

FIG. 5. Deposit of calcium carbonate precipitated by ammonium carbonate in distilled water containing 0.1 per cent. lecithin. Spherules of various sizes, a marked tendency to fusion, and formation of large irregular masses are to be noted.

FIG. 6. Deposit of calcium carbonate precipitated by potassium carbonate in distilled water containing 0.1 per cent. lecithin. Note a tendency to clumping, various sizes of spherules with some double forms. The last three forms indicate one process of disintegration of a spherule, by the occurrence of radial lines along which it splits into sectors, which thus become triangular crystals.

FIG. 7. Deposit of calcium carbonate precipitated by sodium carbonate in a solution of egg albumen. Practically the whole precipitate was in form of spherules.

FIG. 8. Deposit of calcium carbonate precipitated by ammonium carbonate in a solution of egg albumen. Spherules of varying size are seen, also some crystals.

FIG. 9. Deposit of calcium carbonate precipitated by potassium carbonate in a solution of egg albumen. Many spherules are seen, and a tendency towards fusion is noted.

FIG. 10. Deposit of calcium carbonate precipitated by sodium carbonate in a solution of egg albumen containing 0.1 per cent. lecithin. Note the markedly increased size of spherules and crystals and the tendency to fusion.

FIG. 11. Deposit of calcium carbonate precipitated by ammonium carbonate in a solution of egg albumen containing 0.1 per cent. lecithin. Note the increased size of particles, and their tendency to fuse.

FIG. 12. Deposit of calcium carbonate precipitated by potassium carbonate in a solution of egg albumen containing 1 per cent. of acetone. Crystals and spherules both occur. One crystal is seen fused with a spherule.

FIG. 13. Deposit of calcium carbonate by interaction of calcium chloride in a 1 per cent. gelatin solution with sodium carbonate in a 4 per cent. gelatin.

Fields are shown by various sized spherules, of crystals, and of spherules and crystals mixed.

FIG. 14. Deposit of calcium carbonate by interaction of calcium chloride in 1 per cent. gelatin solution with ammonium carbonate in 4 per cent. gelatin solution. Spherules of various sizes are seen.

FIG. 15. Deposit of calcium carbonate by interaction of calcium chloride in 1 per cent. gelatin solution containing 0.1 per cent. lecithin, with ammonium carbonate in 4 per cent. gelatin solution containing 0.1 per cent. lecithin. Note the increase in size of the spherules over those shown in Fig. 14. Three different degrees of fusion are illustrated in the double forms.

FIG. 16. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of untreated gelatin.

FIG. 17. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $1/16$ HCl solution. Large crystalline precipitate.

FIG. 18. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $1/32$ HCl solution. Large, markedly crystalline deposit.

FIG. 19. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $1/512$ HCl solution. The field shown in this figure was composed for some months altogether of clear spherules and a few dumb-bells. The spherules here illustrated are now undergoing disintegration and in their place are appearing large crystals. Some debris is seen in one corner. One spherule is seen partly from the side, showing its true shape to be a disc.

FIG. 20. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $1/2048$ HCl solution. Disintegrating spherules and new crystals are seen.

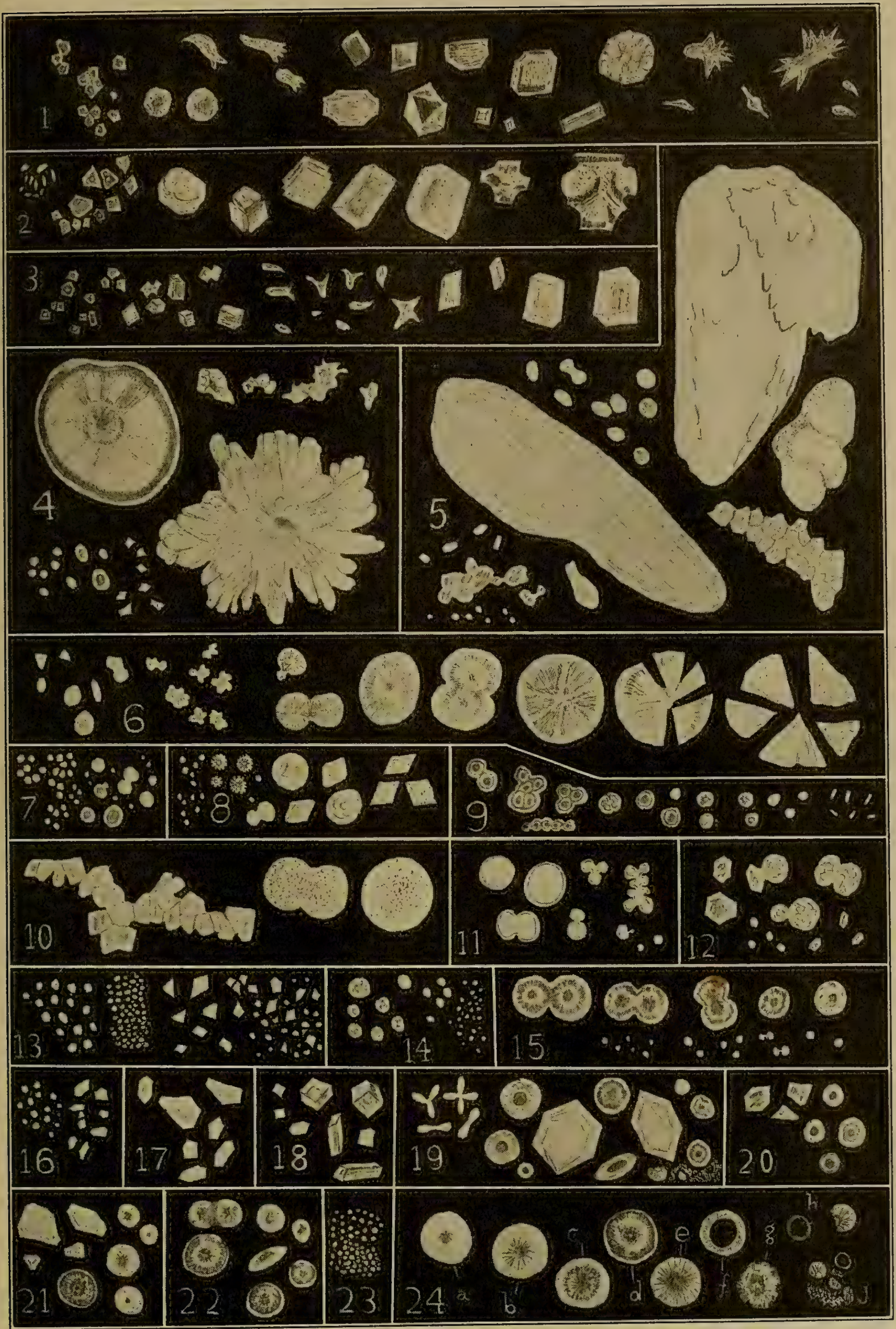
FIG. 21. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $M/8192$ NaOH solution. Newly formed crystals and various stages of disintegrating spherules are shown.

FIG. 22. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $M/4096$ NaOH solution.

FIG. 23. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $M/2048$ NaOH solution. Precipitate is all in the form of small spherules which have been in constant Brownian movement for six months.

FIG. 24. A series of spherules showing various stages in the disintegrative changes which occur before they redissolve. *a*, Spherule with small central opaque area. *b*, Spherule with opaque radial striation extending from central spot. *c*, Spherule with further extension of opaque area. *d*, Spherule almost completely opaque. Narrow, bright, clear periphery. *e*, Spherule striated right out to periphery. *f*, Spherule like that in *d*, dissolving both at periphery and in center in a regular manner. *g*, Spherule like that in *e*, dissolving at an irregular rate at the periphery. *h*, *j*, Remains of dissolving spherules, and debris of some that have fallen apart.

PLATE I.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J. C. WATT.

PLATE 2.

Photomicrographs all taken at a standard magnification, × 200, with dark field condenser in use.

FIG. 25. Crystals of calcium carbonate precipitated by ammonium carbonate in water.

FIG. 26. Calcium carbonate precipitated by sodium carbonate in a solution of egg albumen containing $N/10$ sodium chloride.

FIG. 27. Calcium carbonate precipitated by potassium carbonate in water containing 0.1 per cent. lecithin. Note one double spherule and three large single spherules of which the two larger show indications of the radial divisions which will result in splitting the spherule into crystals of triangular outline. Many small crystals and spherules seen.

FIG. 28. Calcium carbonate precipitated by ammonium carbonate in water containing 0.1 per cent. lecithin.

FIG. 29. Calcium carbonate precipitated by potassium carbonate in a solution of egg albumen containing 1 per cent. ethyl alcohol.

FIG. 30. Calcium carbonate precipitated by potassium carbonate in a solution of egg albumen containing 0.05 per cent. urea.

FIG. 31. Calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with 1/1024 HCl solution. Clear spherules, some seen from side appearing disc-like.

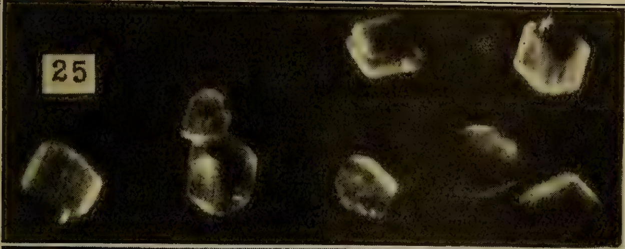
FIG. 32. Calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with 1/2048 HCl solution. Some crystals are present. Spherules begin to show an opaque central dot, the first indication of a coming disintegration.

FIG. 33. Calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with 1/512 HCl solution. This field was formerly all spherules, now some crystals are seen. Spherules are far advanced in disintegrative process, are completely opaque, and are dissolving, some appearing only as ghosts of their former selves.

FIG. 34. Calcium carbonate precipitated by ammonium carbonate in 1 per cent. gelatin solution containing 0.05 per cent. urea. Rings and striations seen in spherules.

FIG. 35. Calcium carbonate precipitated by sodium carbonate in a 1 per cent. gelatin solution containing 0.05 per cent. urea. Field of very small mixed crystals and spherules.

FIG. 36. Combined precipitate of calcium phosphate and calcium carbonate by action of sodium phosphate and sodium carbonate in dog's blood serum. The calcium carbonate shows as seven spherules. The calcium phosphate shows as a cloud of very fine amorphous granules, which is the form in which it precipitated in all experiments.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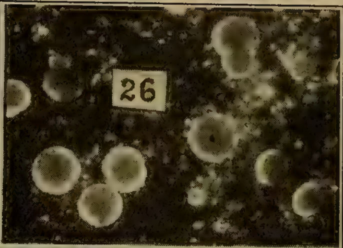
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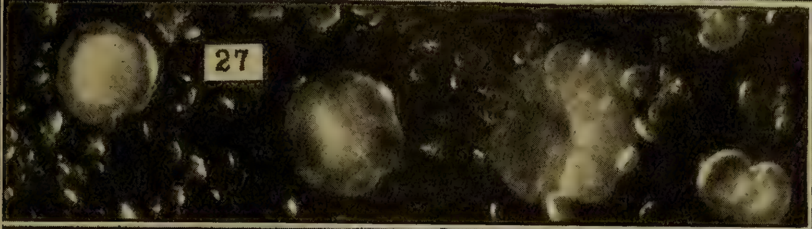
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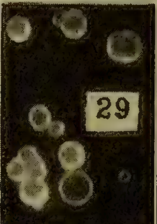
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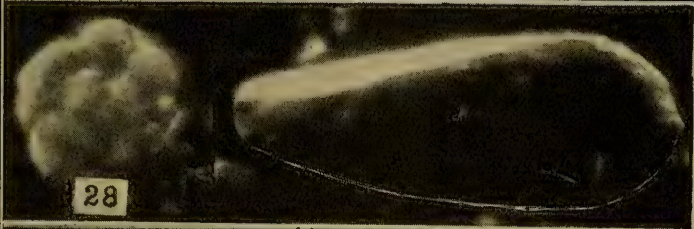
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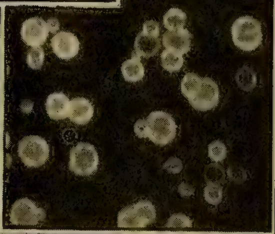

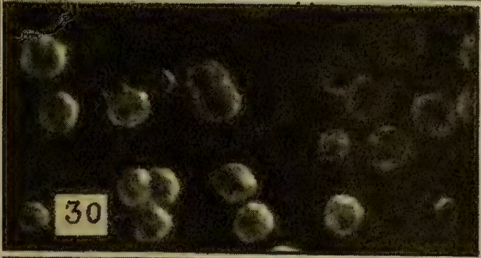
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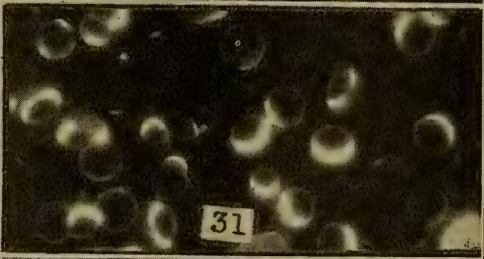
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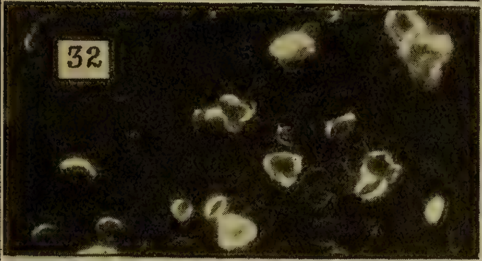
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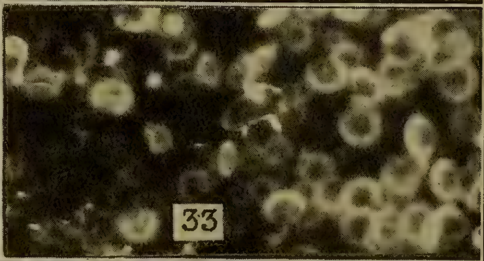
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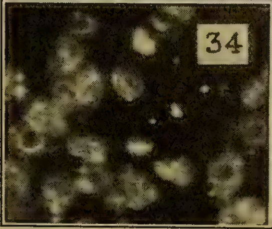
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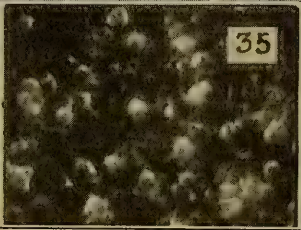
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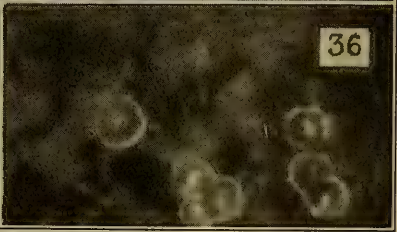
36

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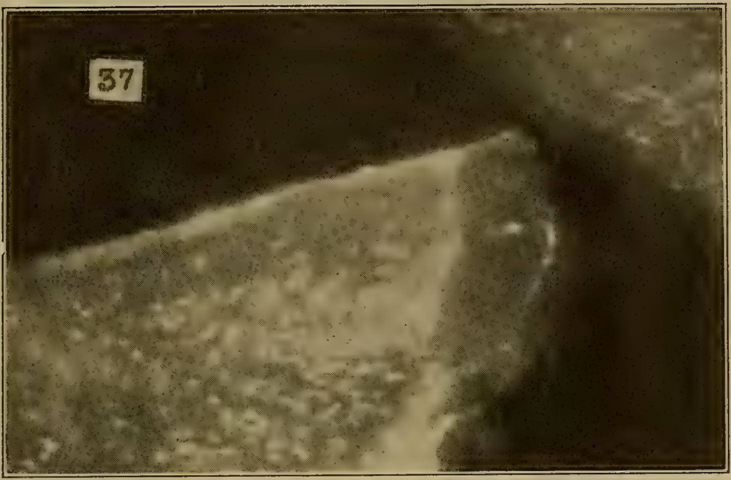
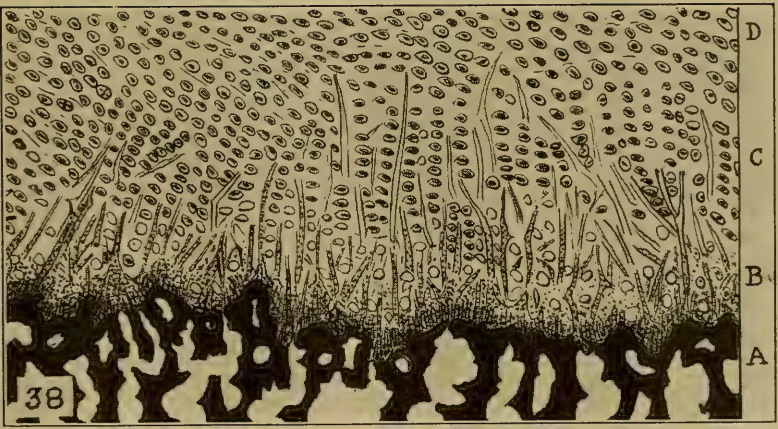
PLATE 3.

FIG. 37. Photomicrograph of a small piece of bone from the growing femur of a mouse. The bone cells show as small, oval, opaque bodies in the specimen. No discrete particles can be seen in the matrix. Magnification, × 200.

FIG. 38. Outline sketch made with camera lucida, of the growing bony end of the diaphysis of the humerus of a six months human foetus. Four zones can be distinguished. *A,* fully formed bone spicules (shown as solid black, marrow spaces white, bone and marrow cells not indicated) with calcium salts fused. *B,* osteogenic fibres, impregnated with calcium salts in form of minute granules. *C,* newly forming osteogenic fibres, not yet impregnated with bone salts, and cartilage showing changes preparatory to bone formation. *D,* unaltered cartilage. Magnification, × 300.





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